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(54) Title: AMYLOID β PROTEIN (GLOBULAR ASSEMBLY AND USES THEREOF)

(57) Abstract: The invention provides amyloid beta-derived dementing ligands (ADDLs) that comprise amyloid β protein assembled into globular non-fibrillar oligomeric structures capable of activating specific cellular processes. The invention also provides methods for assaying the formation, presence, receptor protein binding and cellular activity of ADDLs, as well as compounds that block the formation or activity of ADDLs, and methods of identifying such compounds. The invention further provides methods of using ADDLs, and modulating ADDL formation and/or activity, *inter alia* in the treatment of learning and/or memory disorders.

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En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

AMYLOID β PROTEIN (GLOBULAR ASSEMBLY AND USES THEREOF)**GOVERNMENT RIGHTS IN THE INVENTION**

5 The invention was made with government support under Agreement Nos. AG15501-02, AG-13496-02, AG10481-02, NS34447, and AG13499-03, awarded by the National Institutes of Health. Accordingly, the government may have certain rights in the invention.

RELATED APPLICATIONS

10 This is a continuation-in-part application of U.S. Patent Application Serial No. 08/796,089, filed February 5, 1997, now allowed, U.S. Patent Application Serial No. 60/095,264, filed August 4, 1998, and PCT Application PCT/US98/02426, filed February 5, 1998, still pending.

TECHNICAL FIELD OF INVENTION

15 The present invention pertains to a new composition of matter, amyloid beta-derived dementing ligands (ADDLs). ADDLs comprise amyloid β peptide assembled into soluble globular non-fibrillar oligomeric structures that are capable of activating
20 specific cellular processes. The invention also provides methods for assaying the formation, presence, receptor protein binding and cellular activities of ADDLs. Also described are compounds that block the formation or activity of ADDLs, and methods of identifying such compounds. ADDL formation and activity is relevant *inter alia* to learning and memory. Modulation of ADDL formation or activity thus can be
25 employed according to the invention in the treatment of learning and memory disorders, as well as other diseases, disorders or conditions that are due to the effects of the ADDLs.

BACKGROUND OF THE INVENTION

Alzheimer's disease is a progressive neurodegenerative disease, characterized by distinct pathologies, including neurofibrillary tangles, neuritic plaques, neuronal atrophy, dendritic pruning and neuronal death. From a historical perspective,

5 definitive diagnosis of Alzheimer's disease always has relied upon identification of specific pathologic hallmarks, namely the neurofibrillary tangles which represent the collapsed cytoskeleton of dead and dying neurons, and neuritic plaques, which are extracellular deposits of various protein, lipid, carbohydrate and salt compounds, the primary protein component of which is a 39-43 residue peptide known as amyloid β .

10 From the standpoint of disease impact, however, it is the symptoms manifest in Alzheimer's disease, namely the loss of memory, the erosion of cognitive functions, and the significant changes in personality and behavior, which are most significant. Underlying these symptomatic changes are specific cellular mechanisms that cause nerve cells to malfunction, and eventually to degenerate and die. These
15 cellular mechanisms undoubtedly operate within a background environment that variously affords some level of protection, or exerts contributing and exacerbating effects. The result is a very broad age/incidence distribution curve, with few clues from population studies that point to specific causes.

Molecular genetics represents one realm of study where a clear picture of
20 familial Alzheimer's disease is emerging. As described in more detail below, it is now clear from studies identifying mutations in 3 different proteins, APP and the presenilins 1 and 2, that the final common pathway leading to Alzheimer's disease is the increased production of amyloid β 1-42 (as well as amyloid β 1-43), which occurs in all of these different familial AD mutations. This is particularly noteworthy,
25 because ADDLs, the central focus of the invention described herein, only form as stable entities from this longer form of amyloid, and not from the more prevalent, shorter form A β 1-40.

Amyloid β in Alzheimer's Disease. In 1984, Glenner and Wong succeeded in

isolating and identifying the cerebrovascular amyloid associated with Alzheimer's disease (Glenner et al., *Biochem. Biophys. Res. Commun.*, 120, 885-890, 1984a). Subsequently, the same 39-43 residue peptides now known as amyloid β were identified as the major protein component of Alzheimer's disease neuritic plaques

5 (Glenner et al., *Biochem. Biophys. Res. Commun.*, 122, 1131-1135 1984b; Masters et al., *EMBO J.*, 4, 2757-2764, 1985a; Masters et al., *Proc. Natl. Acad. Sci.*, 82, 4245-4249, 1985b). This was the first time a discrete molecule had been linked to Alzheimer's disease, a disease which to that point had been characterized only by neuroanatomy and neuropathology descriptions. Amyloid β also was identified as the

10 plaque component in brains of Down's Syndrome individuals, (Glenner et al., *Biochem. Biophys. Res. Commun.*, 122, 1131-1135, 1984b; Masters et al., *EMBO J.*, 4, 2757-2764, 1985a; Masters et al., *Proc. Natl. Acad. Sci.*, 82, 4245-4249, 1985b) leading to the suggestion that the gene encoding it might exist on chromosome 21. By 1987, a number of groups had used the amyloid β sequence information and molecular

15 genetics techniques to validate that suggestion, identifying the gene for the amyloid precursor protein (APP) (Kang et al., *Nature*, 325, 733, 1987; Tanzi et al., *Science*, 235, 880-884, 1987).

The APP gene is a large, multi-exon gene that is differentially spliced into a number of APP's (reviewed in Selkoe, In, *Annual Review of Neuroscience*, Cowan

20 (Ed.), 17, ix + 623 p, 489-517, 1994). The proteins are large transmembrane proteins, now known to be processed by several pathways, one or more of which may generate amyloid β . The earliest studies of APP processing had suggested that amyloid β formation was not a normal process (Esch et al., *Science*, 248, 1122-1124 1990; Sisodia et al., *Science*, 248, 492-495, 1990), though subsequent studies in cultured

25 cells and analysis of serum and cerebrospinal fluid have shown that amyloid β formation occurs as a normal process in many cell types, though its formation may not represent a predominant overall pathway.

Pivotal genetic studies of DNA from individuals afflicted with early onset of

familial Alzheimer's disease revealed that mutations in a single gene, this same APP gene, were causative for this very severe form of the disease. Interestingly, several different mutations in the APP gene were found including three different single residue substitutions at Val 717, four residues downstream of the amyloid β 1-42 C-terminus (Goate et al., *Nature*, 349, 704-6 1991; Chartier-Harlin et al., *Nature*, 353, 844-6 1991; Murrell et al., *Science*, 254, 97-9, 1991), and a two residue mutation (670-671) immediately upstream of the amyloid β N-terminus, associated with early onset familial Alzheimer's disease in a Swedish family (Mullan et al., *Nature Genetics* 1, 345-347, 1992). When a vector encoding the cDNA of the Swedish mutant APP gene was transfected into cell lines to evaluate APP processing, it was found that six-eight times more amyloid β was formed, when compared with levels from wild-type APP (Citron et al., *Nature*, 360, 672-674, 1992; Cai et al., *Science*, 259, 514-516, 1993). It was also demonstrated that brain tissue extracts containing native human brain protease activities were able to process a fluorogenic octapeptide substrate encompassing the Swedish mutation more than 100-fold faster than the corresponding substrate based on the wild-type sequence (Ladror et al., *J. Biol. Chem.*, 269, 18422-8, 1994). These results suggest that the mechanism by which the Swedish mutation causes early onset familial Alzheimer's disease involves substantial overproduction of amyloid β . Similar studies of amyloid formation in cells transfected with the 717 mutant APP also had been conducted, but the levels of amyloid β produced were not different from levels produced by wild-type APP. This led to mechanistic speculations that something other than amyloid β production was pathogenic for these mutations. A closer evaluation of processing of the APP 717 mutant, and the Swedish mutant APP by Younkin and co-workers (Suzuki et al., *Science*, 264, 1336-1340, 1994) provided a unified picture of these genetic Alzheimer's disease cases. In this study, not only were total levels of amyloid β production evaluated, but the specific lengths of the amyloid β peptides produced were also analyzed. The results confirmed that the 717 mutation led to more than a

doubling of the ratio of amyloid β 1-42 to amyloid β 1-40 (a highly soluble peptide under physiologic conditions) even though total amyloid β levels did not change. The recently discovered presenilin 1 and 2 familial Alzheimer's disease mutations in genes residing on chromosome 14 (Sherrington et al., *Nature*, 375, 754-758, 1995) and
5 chromosome 1 (Levy-Lahad et al., *Science*, 269, 970-973, 1995), respectively, have also been linked to significant overproduction of amyloid β 1-42. (Mann et al., *Annals of Neurology*, 40, 149-56, 1996; Schuener et al., *Nature Medicine*, 2, 864-70, 1996). Based on these findings, it appears that the pathogenic process mediated by these distinctly different familial Alzheimer's disease mutations is the production of
10 greater levels of amyloid β 1-42. This is the form of amyloid that aggregates most readily (Snyder et al., *Biophys. J.*, 67, 1216-28, 1994), that seeds aggregation of amyloid β to form neuritic plaques (Roher et al., *Neurochem.*, 61, 1916-1926, 1993; Tamaoka et al., *Biochem. Biophys. Res. Commun.*, 205, 834-842, 1994), and, as described herein, the form which unexpectedly forms stable higher order assemblies
15 termed "ADDLs".

Non-amyloid Plaque Components in Alzheimer's Disease. Amyloid β is the major protein component of plaques, comprising more than 70% of the total protein. A variety of other protein components also are present, however, including α 1-
antichymotrypsin (ACT), heparin sulfate proteoglycans (HSPG), apolipoproteins E
20 and J, butyrylcholinesterase (BChE), S-100B, and several complement components. While the importance of these components in the onset and progression of Alzheimer's disease has not been established, involvement of apo E isoforms in the disease has been established by genetic studies of Roses and colleagues (Strittmatter et al., *Proc. Natl. Acad. Sci. USA*, 90, 1977-81, 1993), who discovered that a
25 polymorphism in the apolipoprotein E gene, namely apo E4, correlated with earlier onset of Alzheimer's disease in a large set of late-onset familial Alzheimer's disease cases. Subsequent studies have confirmed that groups of individuals with apo E4 have a significantly greater risk of Alzheimer's disease and that the onset of

Alzheimer's disease roughly parallels the gene dosage for apo E4. On a mechanistic level, studies have revealed that apo E4 binds with lower affinity to amyloid β than apo E3 or apo E2, isoforms which are associated with later onset of Alzheimer's disease. It has been suggested that these isoforms may exert a protective effect by
5 more effective clearance of amyloid β 1-42 deposits (Ladu et al., *J. Biol. Chem.*, 269, 23403-23406, 1994; Ladu et al., *J. Biol. Chem.*, 270, 9039-42, 1995).

The role of other plaque components is not as clear, though recent studies (Oda et al., *Exptl. Neurology*, 136, 22-31, 1995) have shown that apo J (clusterin) can significantly enhance the toxicity of aggregated amyloid β 1-42 *in vitro*. It also has
10 been reported that HSPG enhances the toxicity of amyloid β 1-40 when injected into rat brain (Snow et al., *Soc. Neurosci. Abstr.*, 18, 1465, 1992). Wright et al. (*Ann Neurol.*, 34, 373-384, 1993) demonstrated that amyloid plaques from Alzheimer's disease brain contain significant levels of BChE, while amyloid plaques from elderly non-demented individuals do not. The acute phase inflammatory protein ACT also is
15 upregulated in Alzheimer's disease brain, and it is known to associate with the N-terminal 16 residues of amyloid β . Ma et al. (Ma et al., *Nature*, 372, 92-94, 1994) have reported that ACT can enhance the aggregation of amyloid β 1-42, and these authors speculate that the enhanced aggregation contributes to its neurotoxicity.

Amyloid β Cellular Responses and *In Vivo* Pathology. Beyond the plaques
20 and tangles that are the hallmarks of Alzheimer's disease, it is clear that a range of cellular responses has been induced, both in neurons and in accompanying glial cells. At a biochemical level, hyperphosphorylation of the tau protein is evident, resulting from perturbation of the kinase/phosphatase balance. At a transcriptional level, a variety of genes are activated to produce a spectrum of proteins not normally present
25 or only present at lower levels in the brain. There also is significant evidence that inflammatory processes have been activated. In particular, tau phosphorylation has been documented to be induced by aggregated amyloid β 1-42 in differentiated SH-SY5Y cells (Lambert et al., *J. Neurosci. Res.*, 39, 377-384, 1994), and this result has

been confirmed in a more recent report by Busciglio et al. (*Neuron*, 14, 879-88, 1995), in which amyloid β activated tau phosphorylation in cultured primary rat hippocampal neurons.

Fibrillar Amyloid β and Neurodegeneration in Alzheimer's Disease. The

5 mechanism by which amyloid β 1-42 causes Alzheimer's disease has not been elucidated, but the literature contains more than 200 studies of amyloid β neurotoxicity, many of which have been reviewed recently (e.g., Yankner et al., *Neuron*, 16, 921-32, 1996; Iversen et al., *Biochemical Journal*, 311, 1-16, 1995). The consensus view is that in order for amyloid β to be toxic, it must assemble into
10 fibrillar structures (Pike et al., *J. Neurosci.*, 13, 1676-87, 1993). Solutions containing only monomeric amyloid β have repeatedly been demonstrated to have no deleterious effect on neurons in culture. Furthermore, studies have correlated the formation of amyloid β -sheet containing fibrils and the timing and extent of toxicity using techniques such as circular dichroism and electron microscopy (Simmons et al.,
15 *Molecular Pharmacology*, 45, 373-9, 1994). One study concluded explicitly that amyloid β must exist in fibrillar form in order for it to be toxic (Lorenzo et al., *Proc. Natl. Acad. Sci. USA*, 91, 12243-12247, 1994). Despite this consensus regarding amyloid β structure and activity, there continues to be a problem of reproducibility of published experimental work involving amyloid toxicity (Brining, *Neurobiology of*
20 *Aging*, 18, 581-589, 1997), and widespread variability of activity obtained with different batches of amyloid, or even the same batch of amyloid handled in slightly different ways, in spite of identical chemical composition (May et al., *Neurobiology of Aging*, 13, 1676-87, 1993). This has raised questions regarding the precise structures of amyloid β that are responsible for its activity.

25 The present invention seeks to overcome the problems in the prior art. Accordingly, it is an object of the present invention to provide a new composition of matter, amyloid β peptide assembled into soluble globular non-fibrillar oligomeric structures (ADDLs), that unexpectedly are neurotoxic. These and other objects and

advantages of the present invention, as well as additional inventive features, will be apparent from the following description.

BRIEF DESCRIPTION OF THE FIGURES

5 **Figure 1** is a computer-generated image of a densitometer-scanned silver-stained polyacrylamide gel which shows the ADDLs electrophoresing with a primary band corresponding to about 30 kD, a less abundant band corresponding to about 17 kD, and no evidence of fibrils or aggregates.

Figure 2 is a computer-generated image of a densitometer-scanned
10 Coomassie-stained SDS-polyacrylamide gel which shows ADDLs electrophoresing with a primary band (upper doublet) corresponding to a size of about 17 to about 22 kD, and with another band (lower dark band) indicating abundant 4 kD monomer present, presumably a breakdown product. *Lanes:* first, molecular size markers; second ADDL preparation; third, heavier loading of ADDL preparation.

15 **Figure 3** is a representative computer-generated image of AFM analysis of ADDL-containing "fraction 3" (fractionated on a Superdex 75 gel filtration column).

Figure 4 is a computer-generated image of a densitometer-scanned Coomassie-stained SDS-polyacrylamide gradient gel of ADDLs prepared by
20 coinubation with clusterin (*lane A*) or cold F12 media (*lane B*), and of ADDLs prepared by coinubation with clusterin and which passed through a Centricon 10 kD cut-off membrane (*lane C*) or were retained by a Centricon 10 kD cut-off membrane (*lane D*); MW, molecular size markers.

Figure 5 is a graph of ADDL concentration measured as amyloid β 1-42
25 concentration (nM) vs. % dead cells for brain slices from mice treated with the ADDL preparations.

Figure 6 is a bar chart showing % MTT reduction for control PC 12 cells not exposed to ADDLs ("Cont."), PC 12 cells exposed to clusterin alone ("Apo J"), PC 12 cells exposed to monomeric $A\beta$ ("A β "), PC12 cells exposed to amyloid β

coaggregated with clusterin and aged one day ("A β :Apo J").

Figure 7 is a FACScan showing fluorescence intensity (0-170) versus events (0-300) for B103 cells not exposed to ADDLs (unshaded peak) and B103 cells bound to fluorescent labeled ADDLs (shaded peak).

5 Figure 8 is a FACScan showing fluorescence intensity (0-200) versus events (0-300) for hippocampal cells not exposed to ADDLs (unshaded peak, "-ADDLs") and hippocampal cells bound to fluorescent labeled ADDLs (shaded peak, "+ADDLs").

Figure 9 is a bar chart of percent maximum ADDL binding or ADDL-evoked
10 death for B103 cells that either have been not exposed ("-") or coexposed ("+") to the peptides released by trypsinization of B103 cells.

Figure 10 is a graph of relative ADDL concentration vs. % dead cells for brain
slices from mice treated with the ADDL preparations. To determine relative
concentration, an initial concentration of 10 μ M A β protein was employed to form
15 ADDLs at the highest data point (point "16"), this was subsequently diluted to 1/2
(point "8"), 1/4 (point "4"), and the like.

Figure 11 is a bar chart showing optical density obtained in the ADDL
binding ELISA assay wherein B103 cells were coincubated with ADDLs and 6E10
antibody ("cells, ADDL, 6E10" bar), B103 cells were coincubated with ADDLs and
20 ("cells, ADDL" bar), B103 cells were coincubated with 6E10 antibody ("cells, 6E10"
bar); B103-cells were incubated alone ("cells" bar), 6E10-antibody was incubated
alone ("6E10" bar), or the optical density of diluent was read ("blank" bar).

Figure 12 is a bar chart of % dead cells in either *fyn* +/+ (wild type, "Fyn +";
crosshatched bars) or *fyn* -/- (knockout, "Fyn -"; solid bars) mice either not treated
25 ("Medium") or contacted with ADDLs ("ADDLs").

Figure 13 is a graph of A β concentration (μ M) versus activated glia (number)
obtained upon incubation of astrocytes with ADDLs (filled triangles) or A β 17-42
(filled squares).

Figure 14 is a graph of time (minutes) versus % baseline cell body spike amplitude for control mice not treated with ADDLs (filled triangles) or mice treated with ADDLs (filled squares).

Figure 15 is a graph of time (minutes) versus mean spike amplitude for control rat hippocampal slices not exposed to ADDLs (filled triangles) versus rat hippocampal slices exposed to ADDLs (filled squares).

Figure 16 is a computer-generated image of a densitometer-scanned 16.5% tris-tricine SDS-polyacrylamide gel (Biorad) which shows a range of oligomeric, soluble ADDLs (labeled "ADDLs"), and amyloid β dimer (labeled "Dimer"), and monomer (labeled "Monomer"). Lanes: first, silver stained Mark XII molecular weight standards (Novex, San Diego, California); second, silver stained ADDLs; third, Western blot of second lane using the monoclonal antibody 26D6 (Sibia Neurosciences, San Diego, California).

Figure 17 is a computer-generated image of an AFM analysis of ADDLs. The top view subtracted image shows a high magnification view ($2.0 \mu\text{m} \times 2.0 \mu\text{m}$) of aggregated amyloid β molecules that have been spotted on freshly cleaved mica.

SUMMARY OF THE INVENTION

The invention encompasses a new composition of matter, termed amyloid beta-derived dementing ligands or amyloid beta-derived diffusible ligands (ADDLs). ADDLs consist of amyloid β peptide assembled into soluble non-fibrillar oligomeric structures that are capable of activating specific cellular processes. Another aspect of the invention consists of methods for assaying the formation, presence, receptor protein binding and cellular activities of ADDLs. The invention further encompasses assay methods and methods of identifying compounds that modulate (e.g., increase or decrease) the formation and/or activity of ADDLs. Such compounds can be employed in the treatment of diseases, disorders, or conditions due to the effects of the ADDLs.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that in neurotoxic samples of amyloid β not only do fibrillar structures exist, but also, unexpectedly, some globular protein structures exist that appear to be responsible for the neurotoxicity. Using novel methods, samples that contain predominantly these soluble globular protein assemblies and no fibrillar structures have been generated as described herein. In heterogeneous samples prepared by various methods, the removal of the larger, fibrillar forms of amyloid β by centrifugation does not remove these soluble globular assemblies of amyloid β in the supernatant fractions. These supernatant fractions exhibit significantly higher neurotoxicity than unfractionated amyloid β samples aggregated under literature conditions. These novel and unexpected neurotoxic soluble globular forms are referred to herein as amyloid β -derived dementing ligands, amyloid β -derived diffusible ligands (ADDLs), amyloid β soluble non-fibrillar amyloid β oligomeric structures, or simply oligomeric structures. Samples of amyloid β that had been "aged" under standard literature conditions (e.g., Pike et al., *J. Neurosci.*, **13**, 1676-1687, 1993) for more than three weeks lose their neurotoxicity, even though these samples contain predominantly fibrillar structures with few or no ADDLs. This discovery that the globular ADDLs are neurotoxic is particularly surprising since current thinking holds that it is fibril structures that constitute the toxic form of amyloid β (Lorenzo et al., *Proc. Natl. Acad. Sci. USA*, **91**, 12243-12247, 1994; Howlett et al., *Neurodegen*, **4**, 23-32, 1995).

The ADDLs can be formed *in vitro*. When a solution (e.g., a DMSO solution) containing monomeric amyloid β 1-42 (or other appropriate amyloid β , as further described herein) is diluted into cold tissue culture media (e.g., F12 cell culture media), then allowed to incubate at about 4°C for from about 2 to about 48 hours and centrifuged for about 10 minutes at about 14,000g at a temperature of 4°C, the supernatant fraction contains small, soluble oligomeric globules that are highly

neurotoxic, e.g., in neuronal cell and brain slice cultures. The ADDLs also can be formed by coincubation of amyloid β with certain appropriate agents, e.g., clusterin (a senile plaque protein that also is known as ApoJ), as well as by other methods, as described herein.

5 Thus, in particular, the present invention provides an isolated soluble non-fibrillar amyloid β oligomeric structure. The oligomeric structure so isolated does not contain an exogenous added crosslinking agent. The oligomeric structure desirably is stable in the absence of any crosslinker.

Atomic force microscope analysis (AFM) can be carried out as is known in the art and described herein, for instance, using a Digital Instruments Atomic force microscope as described in Example 3. AFM of such a supernatant fraction (i.e., a supernatant fraction in which fibrillar structures have been removed) reveals a number of different size globules (i.e., or different size oligomeric structures) present in the fraction. These globules fall within the range of from about 4.7 to about 11.0 nm, with the major fraction falling within a size range of from about 4.7 nm to about 6.2 nm. There appear to be distinct species of globules falling within this size range and which correspond to specific size oligomeric species such as those indicated by analysis on certain gel electrophoresis systems, as shown in Figures 2 and 16. Slight variation in height surface results from how the particular species are seated on the mica surface at the time of AFM analysis. Despite this slight variation however, there appear to be several predominant sizes of globules in the 4.7-6.2 size range, i.e., from about 4.9 nm to about 5.4 nm, and from about 5.7 nm to about 6.2 nm, that constitute about 50% of the oligomeric structures in a typical sample. There also appears to be a distinct size species of globule having dimensions of from about 5.3 nm to about 5.7 nm. Larger globules from about 6.5 nm to about 11.0 nm appear less frequently, but may possess neurotoxic properties similar to the more prevalent, smaller species. It appears that the globules of dimensions of from about 4.7 nm to about 6.2 nm on AFM comprise the pentamer and hexamer form of oligomeric

amyloid β ($A\beta$) protein. The AFM size globules of from about 4.2 nm to about 4.7 nm appear to correspond to the $A\beta$ tetramer. The size globules of from about 3.4 nm to about 4.0 nm to appear to correspond to trimer. The large globules appear to correspond to oligomeric species ranging in size from about 13 amyloid monomers to about 24 amyloid monomers. The size globules of from about 2.8 nm to about 3.4 nm correspond to dimer (Roher et al., *J. Biol. Chem.*, 271, 20631-20635, 1996). The $A\beta$ monomer AFM size ranges from about 0.8 nm to about 1.8 – 2.0 nm. Monomeric and dimeric amyloid β are not neurotoxic in neuronal cell cultures or in the organotypic brain slice cultures.

10 Thus, the present invention provides an isolated soluble non-fibrillar amyloid β oligomeric structure (i.e., an ADDL) that preferably comprises at from about 3 to about 24 amyloid β protein monomers, especially from about 3 to about 20 amyloid β protein monomers, particularly from about 3 to about 16 amyloid β protein monomers, most preferably from about 3 to about 12 amyloid β protein monomers, and which desirably comprises at from about 3 to about 6 amyloid β protein monomers. As previously described, large globules (less predominant species) appear to correspond to oligomeric species ranging in size from about 13 amyloid β monomers to about 24 amyloid β monomers. Accordingly, the invention provides an isolated soluble non-fibrillar amyloid β oligomeric structure wherein the oligomeric structure preferably comprises trimer, tetramer, pentamer, hexamer, heptamer, octamer, 12-mer, 16-mer, 20-mer or 24-mer aggregates of amyloid β proteins. In particular, the invention provides an isolated soluble non-fibrillar amyloid β protein oligomeric structure wherein the oligomeric structure preferably comprises trimer, tetramer, pentamer, or hexamer aggregates of amyloid β protein. The oligomeric structure of the invention optimally exhibits neurotoxic activity.

The higher order structure of the soluble non-fibrillar amyloid β protein oligomer structure (i.e., the aggregation of monomers to form the oligomeric structure) desirably can be obtained not only from amyloid β 1-42, but also from any

amyloid β protein capable of stably forming the soluble non-fibrillar amyloid β oligomeric structure. In particular, amyloid β 1-43 also can be employed. Amyloid β 1-42 with biocytin at position 1 also can be employed. Amyloid β (e.g., β 1-42 or β 1-43) with a cysteine at the N-terminus also can be employed. Similarly, A β 5 truncated at the amino terminus (e.g., particularly missing one or more up to the entirety of the sequence of amino acid residues 1 through 8 of A β 1-42 or A β 1-43), or A β (e.g., A β 1-42 or 1-43) having one or two extra amino acid residues at the carboxyl terminus can be employed. By contrast, amyloid β 1-40 can transiently form ADDL-like structures which can be toxic, but these structures are not stable and cannot be isolated as aqueous solutions, likely due to the shortened nature of the protein, which limits its ability to form such higher order assemblies in a stable fashion.

Desirably, the isolated soluble non-fibrillar amyloid β oligomeric structure according to the invention comprises globules of dimensions of from about 4.7 nm to about 11.0 nm, particularly from about 4.7 nm to about 6.2 nm as measured by atomic force microscopy. Also, preferably the isolated soluble non-fibrillar amyloid β oligomeric structure comprises globules of dimensions of from about 4.9 nm to about 5.4 nm, or from about 5.7 nm to about 6.2 nm, or from about 6.5 nm to about 11.0 nm, as measured by atomic force microscopy. In particular, preferably the isolated soluble non-fibrillar amyloid β oligomeric structure according to the invention is such that wherein from about 30% to about 85%, even more preferably from about 40% to about 75% of the assembly comprises two predominant sizes of globules, namely, of dimensions of from about 4.9 nm to about 5.4 nm, and from about 5.7 nm to about 6.2 nm, as measured by atomic force microscopy. However, it also is desirable that the oligomeric structure comprises AFM size globules of about 5.3 to about 5.7 nm. It is also desirable that the oligomeric structure may comprise AFM size globules of about 6.5 nm to about 11.0 nm.

By non-denaturing gel electrophoresis, the bands corresponding to ADDLs run

at about from 26 kD to about 28 kD, and with a separate broad band representing sizes of from about 36 kD to about 108 kD. Under denaturing conditions (e.g., on a 15% SDS-polyacrylamide gel), the ADDLs comprise a band that runs at from about 22 kD to about 24 kD, and may further comprise a band that runs at about 18 to about 19 kD.

5 Accordingly, the invention preferably provides an isolated soluble non-fibrillar amyloid β oligomeric structure (i.e., ADDL) that has a molecular weight of from about 26 kD to about 28 kD as determined by non-denaturing gel electrophoresis. The invention also preferably provides an isolated soluble non-fibrillar amyloid β oligomeric structure (i.e., ADDL) that runs as a band corresponding to a molecular
10 weight of from about 22 kD to about 24 kD as determined by electrophoresis on a 15% SDS-polyacrylamide gel. The invention further preferably provides an isolated soluble non-fibrillar amyloid β oligomeric structure (i.e., ADDL) that runs as a band corresponding to a molecular weight of from about 18 kD to about 19 kD as determined by electrophoresis on a 15% SDS-polyacrylamide gel.

15 Also, using a 16.5% tris-tricine SDS-polyacrylamide gel system, additional ADDL bands can be visualized. The increased resolution obtained with this gel system confirms the ability to obtain according to the invention an isolated oligomeric structure having a molecular weight ranging from about 13 kD to about 116 kD, as determined by electrophoresis on a 16.5% tris-tricine SDS-polyacrylamide gel. The
20 ADDL bands appear to correspond to distinct size species. In particular, use of this gel system allows visualization of bands corresponding to trimer with a size of about 13 to about 14 kD, tetramer trimer with a size of about 17 to about 19 kD, pentamer with a size of about 22 kD to about 23 kD, hexamer with a size of about 26 to about 28 kD, heptamer with a size from about 32 kD to 33 kD, and octamer with a size from
25 about 36 kD to about 38 kD, as well as larger soluble oligomers ranging in size from about 12 monomers to about 24 monomers. Thus, the invention desirably provides an isolated oligomeric structure, wherein the oligomeric structure has, as determined by electrophoresis on a 16.5% tris-tricine SDS-polyacrylamide gel, a molecular weight

selected from the group consisting of from about 13 kD to about 14 kD, from about 17 kD to about 19 kD, from about 22 kD to about 23 kD, from about 26 kD to about 28 kD, from about 32 kD to about 33 kD, and from about 36 kD to about 38 kD.

The invention further provides a method for preparing the isolated soluble
5 non-fibrillar amyloid β oligomeric structure. This method optionally comprises the steps of:

- (a) obtaining a solution of monomeric amyloid β protein;
- (b) diluting the protein solution into an appropriate media;
- (c) incubating the media resulting from step (b) at about 4°C;
- 10 (d) centrifuging the media at about 14,000 g at about 4°C; and
- (e) recovering the supernatant resulting from the centrifugation as containing the amyloid β oligomeric structure. In step (c) of this method, the solution desirably is incubated for from about 2 hours to about 48 hours, especially from about 12 hours to about 48 hours, and most preferably from about 24 hours to about 48
15 hours. In step (d) of this method, the centrifugation preferably is carried out for from about 5 minutes to about 1 hour, especially for from about 5 minutes to about 30 minutes, and optimally for about 10 minutes. Generally, however, this is just a precautionary measure to remove any nascent fibrillar or protofibrillar structures and may not be necessary, particularly where long-term stability of the ADDL preparation
20 is not an issue.

The A β protein is diluted in step (b) desirably to a final concentration ranging from about 5 nM to about 500 μ M, particularly from about 5 μ M to about 300 μ M, especially at about 100 μ M. The "appropriate media" into which the A β protein solution is diluted in step (b) preferably is any media that will support, if not facilitate,
25 ADDL formation. In particular, F12 media (which is commercially available as well as easily formulated in the laboratory) is preferred for use in this method of the invention. Similarly, "substitute F12 media" also desirably can be employed. Substitute F12 media differs from F12 media that is commercially available or which

is formulated in the laboratory. According to the invention, substitute F12 media preferably comprises the following components: N, N-dimethylglycine, D-glucose, calcium chloride, copper sulfate pentahydrate, iron(II) sulfate heptahydrate, potassium chloride, magnesium chloride, sodium chloride, sodium bicarbonate, disodium
5 hydrogen phosphate, and zinc sulfate heptahydrate.

In particular, synthetic F12 media according to the invention optionally comprises: N, N-dimethylglycine (from about 600 to about 850 mg/L), D-glucose (from about 1.0 to about 3.0 g/L), calcium chloride (from about 20 to about 40 mg/L), copper sulfate pentahydrate (from about 15 to about 40 mg/L), iron(II) sulfate
10 heptahydrate (from about 0.4 to about 1.2 mg/L), potassium chloride (from about 160 to about 280 mg/L), magnesium chloride (from about 40 to about 75 mg/L), sodium chloride (from about 6.0 to about 9.0 g/L), sodium bicarbonate (from about 0.75 to about 1.4 g/L), disodium hydrogen phosphate (from about 120 to about 160 mg/L), and zinc sulfate heptahydrate (from about 0.7 to about 1.1 mg/L). Optimally,
15 synthetic F12 media according to the invention comprises: N, N-dimethylglycine (about 766 mg/L), D-glucose (about 1.802 g/L), calcium chloride (about 33 mg/L), copper sulfate pentahydrate (about 25 mg/L), iron(II) sulfate heptahydrate (about 0.8 mg/L), potassium chloride (about 223 mg/L), magnesium chloride (about 57 mg/L), sodium chloride (about 7.6 g/L), sodium bicarbonate (about 1.18 g/L), disodium
20 hydrogen phosphate (about 142 mg/L), and zinc sulfate heptahydrate (about 0.9 mg/L). Further, the pH of the substitute-F12 media preferably is adjusted, for instance, using 0.1 M sodium hydroxide, desirably to a pH of from about 7.0 to about 8.5, and preferably a pH of about 8.0.

The foregoing method further desirably can be carried out by forming the
25 slowly-sedimenting oligomeric structure in the presence of an appropriate agent, such as clusterin. This is done, for instance, by adding clusterin in step (c), and, as set out in the Examples which follow.

Moreover, the invention also provides as described in the Examples, a method

for preparing a soluble non-fibrillar amyloid β oligomeric structure, according to the invention, wherein the method comprises:

- (a) obtaining a solution of monomeric amyloid β protein, the amyloid β protein being capable of forming the oligomeric structure;
- 5 (b) dissolving the amyloid β monomer in hexafluoroisopropanol;
- (c) removing hexafluoroisopropanol by speed vacuum evaporation to obtain solid peptide;
- (d) dissolving the solid peptide in DMSO to form a DMSO stock solution;
- (e) diluting the stock solution into an appropriate media;
- 10 (f) vortexing; and
- (g) incubating at about 4°C for about 24 hours.

If the ADDLs are prepared by the incorporation of 10% biotinylated amyloid β 1-42 (or other appropriate biotinylated amyloid β protein), they can be utilized in a receptor binding assay using neural cells and carried out, for instance, on a

15 fluorescence activated cell sorting (FACS) instrument, with labeling by a fluorescent avidin conjugate. Alternately, instead of incorporating biotin in the amyloid β protein, another reagent capable of binding the ADDL to form a fluorescently labeled molecule, and which may already be part of a fluorescent-labeled conjugate, can be employed. For instance, the soluble non-fibrillar amyloid β oligomeric structure can

20 be formed such that the amyloid protein includes another binding moiety, with "binding moiety" as used herein encompassing a molecule (such as avidin, streptavidin, polylysine, and the like) that can be employed for binding to a reagent to form a fluorescently-labeled compound or conjugate. The "fluorescent reagent" to which the oligomeric structure binds need not itself fluoresce directly, but instead may

25 merely be capable of fluorescence through binding to another agent. For example, the fluorescent reagent which binds the oligomeric structure can comprise a β amyloid specific antibody (e.g., 6E10), with fluorescence generated by use of a fluorescent secondary antibody.

Along with other experiments, FACScan analysis of the rat CNS B103 cells was done without and with ADDL incubation. Results of these and further studies confirm that binding to the cell surface is saturable, and brief treatment with trypsin selectively removes a subset of cell surface proteins and eliminates binding of
5 ADDLs. Proteins that are cleavable by brief treatment with trypsin from the surface of B103 cells also prevent ADDL binding to B103 cells or cultured primary rat hippocampal neurons. These results all support that the ADDLs act through a particular cell surface receptor, and that early events mediated by the ADDLs (i.e., events prior to cell killing) can be advantageously controlled (e.g., for treatment or
10 research) by compounds that block formation and activity (e.g., including receptor binding) of the ADDLs.

Thus, the invention provides a method for identifying compounds that modulate (i.e., either facilitate or block) activity (e.g., activity such as receptor binding) of the ADDL. This method preferably comprises:

- 15 (a) contacting separate cultures of neuronal cells with the oligomeric structure of the invention either in the presence or absence of contacting with the test compound;
- (b) adding a reagent that binds to the oligomeric structure, the reagent being fluorescent;
- 20 (c) analyzing the separate cell cultures by fluorescence-activated cell sorting; and
- (d) comparing the fluorescence of the cultures, with compounds that block activity (i.e., binding to a cell surface protein) of the oligomeric structure being identified as resulting in a reduced fluorescence of the culture, and compounds that
25 facilitate binding to a cell surface protein (i.e., a receptor) being identified as resulting in an increased fluorescence of the culture, as compared to the corresponding culture contacted with the oligomeric structure in the absence of the test compound.

Alternately, instead of adding a fluorescent reagent that in and of itself is able to bind

the protein complex, the method desirably is carried out wherein the oligomeric structure is formed from amyloid β 1-42 protein (or another amyloid β) prepared such that it comprises a binding moiety capable of binding the fluorescent reagent.

Similarly, the method can be employed for identifying compounds that
5 modulate (i.e., either facilitate or block) formation or activity (e.g., binding to a cell surface protein, such as a receptor) of the oligomeric structure comprising:

- (a) preparing separate samples of amyloid β that either have or have not been mixed with the test compound;
- (b) forming the oligomeric structure in the separate samples;
- 10 (c) contacting separate cultures of neuronal cells with the separate samples;
- (d) adding a reagent that binds to the oligomeric structure, the reagent being fluorescent;
- (e) analyzing the separate cell cultures by fluorescence-activated cell
15 sorting; and
- (f) comparing the fluorescence of the cultures, with compounds that block formation or binding to a cell surface protein of the oligomeric structure being identified as resulting in a reduced fluorescence of the culture, and compounds that facilitate formation or binding to a cell surface protein of the oligomeric structure
20 being identified as resulting in an increased fluorescence of the culture, as compared to the corresponding culture contacted with the oligomeric structure in the absence of the test compound. Further, instead of adding a fluorescent reagent that in and of itself is able to bind the protein complex, the method can be carried out wherein the oligomeric structure is formed from amyloid β protein prepared such that it comprises
25 a binding moiety capable of binding the fluorescent reagent.

The fluorescence of the cultures further optionally is compared with the fluorescence of cultures that have been treated in the same fashion except that instead of adding or not adding test compound prior to formation of the oligomeric structure,

the test compound either is or is not added after formation of the oligomeric structure.

In this situation, compounds that block formation of the oligomeric structure are identified as resulting in a reduced fluorescence of the culture, and compounds that facilitate formation of the oligomeric structure are identified as resulting in an increased fluorescence of the culture, as compared to the corresponding culture contacted with the oligomeric structure in the absence of the test compound, *only* when the compound is added prior to oligomeric structure.

By contrast, compounds that block binding to a cell surface protein (e.g., a receptor) of the oligomeric structure are identified as resulting in a reduced fluorescence of the culture, and compounds that facilitate binding to a cell surface protein of the oligomeric structure are identified as resulting in an increased fluorescence of the culture, as compared to the corresponding culture contacted with the oligomeric structure in the absence of the test compound, when the compound is added *either prior to or after* oligomeric structure.

In a similar fashion, a cell-based assay, particularly a cell-based enzyme-linked immunosorbent assay (ELISA) can be employed in accordance with the invention to assess ADDL binding activity. In particular, the method can be employed to detect binding of the oligomeric structure to a cell surface protein. This method preferably comprises:

- (a) forming an oligomeric structure from amyloid β protein;
- (b) contacting a culture of neuronal cells with the oligomeric structure;
- (c) adding an antibody (e.g., 6E10) that binds said oligomeric structure, said antibody including a conjugating moiety (e.g., biotin, or other appropriate agent);
- (d) washing away unbound antibody;
- (e) linking an enzyme (e.g., horseradish peroxidase) to said antibody bound to said oligomeric structure by means of said conjugating moiety;
- (f) adding a colorless substrate (e.g., ABTS) that is cleaved by said enzyme to yield a color change; and

(g) determining said color change (e.g., spectrophotometrically) or the rate of the color change as a measure of binding to a cell surface protein (e.g., a receptor) of said oligomeric structure. As earlier described, the antibody can be any antibody capable of detecting ADDLs (e.g., an antibody directed to an exposed site on amyloid β), and the antibody conjugating moiety can be any agent capable of linking a means of detection (e.g., an enzyme). The enzyme can be any moiety (e.g., perhaps even other than a protein) that provides a means of detecting (e.g., color change due to cleavage of a substrate), and further, can be bound (e.g., covalent or noncovalent) to the antibody bound to the oligomeric structure by means of another moiety (e.g., a secondary antibody). Also, preferably according to the invention the cells are adhered to a solid substrate (e.g., tissue culture plastic) prior to the conduct of the assay. It goes without saying that desirably step (b) should be carried out as described herein such that ADDLs are able to bind to cells. Similarly, preferably step (c) should be carried out for a sufficient length of time (e.g., from about 10 minutes to about 2 hours, desirably for about 30 minutes) and under appropriate conditions (e.g., at about room temperature, preferably with gentle agitation) to allow antibody to bind to ADDLs. Further, appropriate blocking steps can be carried out such as are known to those skilled in the art using appropriate blocking reagents to reduce any nonspecific binding of the antibody. The artisan is familiar with ELISAs and can employ modifications to the assay such as are known in the art.

The assay desirably also can be carried out so as to identify compounds that modulate (i.e., either facilitate or block) formation or binding to a cell surface protein of the oligomeric structure. In this method, as in the prior-described assays for test compounds, the test compound is either added to the ADDL preparation, prior to the contacting of the cells with the ADDLs. This assay thus can be employed to detect compounds that modulate formation of the oligomeric structure (e.g., as previously described). Moreover, the test compound can be added to the ADDL preparation prior to contacting the cells (but after ADDL formation), or to the cells prior to contact with

ADDLs. This method (e.g., as previously described) can be employed to detect compounds that modulate ADDL binding to the cell surface. Also, a test compound can be added to the mixture of cells plus ADDLs. This method (e.g., as previously described) can be employed to detect compounds that impact on ADDL-mediated events occurring downstream of ADDL binding to a cell surface protein (e.g., to an ADDL receptor). The specificity of the compounds for acting on an ADDL-mediated downstream effect can be confirmed, for instance, by simply adding the test compound in the absence of any coincubation with ADDLs. Of course, further appropriate controls (e.g., as set forth in the following Examples and as known to those skilled in the art) should be included with all assays.

Similarly, using the methods described herein (e.g., in the Examples), the present invention provides a method for identifying compounds that block formation of the oligomeric structure of the invention, wherein the method desirably comprises:

- (a) preparing separate samples of amyloid β protein that either have or have not been mixed with the test compound;
- (b) forming the oligomeric structure in the separate samples;
- (c) assessing whether any protein assemblies have formed in the separate samples using a method selected from the group consisting of electrophoresis, immunorecognition, and atomic force microscopy; and
- (d) comparing the formation of the protein assemblies in the separate samples, which compounds that block formation of the oligomeric structure being identified as resulting in decreased formation of the oligomeric structure in the sample as compared with a sample in which the oligomeric structure is formed in the absence of the test compound.

This information on compounds that modulate (i.e., facilitate or block) formation and/or activity including binding to a cell surface protein of the oligomeric structure can be employed in the research and treatment of ADDL-mediated diseases, conditions, or disorders. The methods of the invention can be employed to investigate

the activity and neurotoxicity of the ADDLs themselves. For instance, when 20 nL of the ADDL preparation was injected into the hippocampal region of an adult mouse 60-70 minutes prior to the conduct of a long-term potentiation (LTP) experiment (e.g. Namgung et al., *Brain Research*, 689, 85-92, 1995), the stimulation phase of the experiment occurred in a manner identical with saline control injections, but the consolidation phase showed a significant, continuing decline in synaptic activity as measured by cell body spike amplitude, over the subsequent 2 hours, compared with control animals, in which synaptic activity remained at a level comparable to that exhibited during the stimulation phase. Analysis of brain slices after the experiment indicated that no cell death had occurred. These results, as well as other described in the following Examples, confirm that ADDL treatment compromised the LTP response. This indicates that ADDLs contribute to the compromised learning and memory observed in Alzheimer's disease by interference with neuronal signaling processes, rather than by the induction of nerve cell death.

Additional information on the effects of ADDLs (either in the presence or absence of test compounds that potentially modulate ADDL formation and/or activity) can be obtained using the further assays according to the invention. For instance, the invention provides a method for assaying the effects of ADDLs that preferably comprises:

- (a) administering the oligomeric structure to the hippocampus of an animal;
- (b) applying an electrical stimulus; and
- (c) measuring the cell body spike amplitude over time to determine the long-term potentiation response. The method optionally is carried out wherein the long-term potentiation response of the animal is compared to the long-term potentiation response of another animal treated in the same fashion except having saline administered instead of oligomeric structure prior to application of the electrical stimulus. This method further can be employed to identify compounds that modulate

(i.e., increase or decrease) the effects of the ADDLs, for instance, by comparing the LTP response in animals administered ADDLs either alone, or, in conjunction with test compounds.

Along these lines, the invention provides a method for identifying compounds that modulate the effects of the ADDL oligomeric structure. The method preferably comprises:

- (a) administering either saline or a test compound to the hippocampus of an animal;
- (b) applying an electrical stimulus;
- 10 (c) measuring the cell body spike amplitude over time to determine the long-term potentiation response; and
- (d) comparing the long-term potentiation response of animals having saline administered to the long-term potentiation response of animals having test compound administered. The method further optionally comprises administering oligomeric structure to the hippocampus either before, along with, or after administering the saline or test compound.

Similarly, the present invention provides a method for identifying compounds that modulate (i.e., either increase or decrease) the neurotoxicity of the ADDL protein assembly, which method comprises:

- 20 (a) contacting separate cultures of neuronal cells with the oligomeric structure either in the presence or absence of contacting with the test compound;
- (b) measuring the proportion of viable cells in each culture; and
- (c) comparing the proportion of viable cells in each culture. Compounds that block the neurotoxicity of the oligomeric structure are identified, for example, as resulting in an increased proportion of viable cells in the culture as compared to the corresponding culture contacted with the oligomeric structure in the absence of the test compound. Compounds that increase the neurotoxicity of the oligomeric structure are identified, for example, as resulting in a reduced portion of viable cells in

the culture as compared to the corresponding culture contacted with the oligomeric structure in the presence of the test compound.

The methods of the invention also can be employed in detecting in test materials the ADDLs (e.g., as part of research, diagnosis, and/or therapy). For instance, ADDLs bring about a rapid morphological change in serum-starved B103 cells, and they also activate Fyn kinase activity in these cells within 30 minutes of ADDL treatment (data not shown). ADDLs also induce rapid complex formation between Fyn and focal adhesion kinase (FAK; Zhang et al, *Neurosci. Letters*, 211, 1-4, 1996), and translocating of several phosphorylated proteins and Fyn-Fak complex to a Triton-insoluble fraction (Berg et al., *J. Neurosci. Res.*, 50, 979-989, 1997). This suggests that Fyn and other activated signaling pathways are involved in the neurodegenerative process induced by ADDLs. This has been confirmed by experiments in brain slice cultures from genetically altered mice that lack a functional *fyn* gene, where addition of ADDLs resulted in no increased neurotoxicity compared to vehicle controls.

Therefore, compounds that block one or more of Fyn's function, or Fyn relocation, namely by impacting on ADDLs, may be important neuroprotective drugs for Alzheimer's disease. Similarly, when ADDLs are added to cultures of primary astrocytes, the astrocytes become activated and the mRNA for several proteins, including IL-1, inducible nitric oxide synthase, Apo E, Apo J and α 1-antichymotrypsin become elevated. These phenomena desirably are employed in accordance with the invention in a method for detecting in a test material the ADDL protein assembly. Such methods optionally comprise:

(a) contacting the test material with an antibody (e.g., the 6E10 antibody or another antibody); and

(b) detecting binding to the oligomeric structure of the antibody.

Similarly, the method desirably can be employed wherein

(a) the test material is contacted with serum-starved neuroblastoma cells

(e.g., B103 neuroblastoma cells); and

(b) morphological changes in the cells are measured by comparing the morphology of the cells against neuroblastoma cells that have not been contacted with the test material.

5 The method also preferably can be employed wherein:

(a) the test material is contacted with brain slice cultures; and

(b) brain cell death is measured as compared against brain slice cultures that have not been contacted with the test material. The method further desirably can be conducted wherein:

10 (a) the test material is contacted with neuroblastoma cells (e.g., B103 neuroblastoma cells); and

(b) increases in *fyn* kinase activity are measured by comparing *fyn* kinase activity in the cells against *fyn* kinase activity in neuroblastoma cells that have not been contacted with said test material. In particular, *Fyn* kinase activity can be compared making use of a commercially available kit (e.g., Kit #QIA-28 from Oncogene Research Products, Cambridge, MA) or using an assay analogous to that described in Borowski et al., *J. Biochem. (Tokyo)*, **115**, 825-829, 1994.

In yet another preferred embodiment of the method of detecting ADDLs in test material, the method desirably comprises:

20 (a) contacting the test material with cultures of primary astrocytes; and

(b) determining activation of the astrocytes as compared to cultures of primary astrocytes that have not been contacted with the test material. In a variation of this method, the method optionally comprises:

(a) contacting the test material with cultures of primary astrocytes; and

25 (b) measuring in the astrocytes increases in the mRNA for proteins selected from the group consisting of interleukin-1, inducible nitric oxide synthase, Apo E, Apo J, and α 1-antichymotrypsin by comparing the mRNA levels in the astrocytes against the corresponding mRNA levels in cultures of primary astrocytes

that have not been contacted with the test material. There are, of course, other methods of assay, and further variations of those described above that would be apparent to one skilled in the art, particularly in view of the specification disclosure herein.

5 Thus, clearly, the ADDLs according to the present invention have utility *in vitro*. Such ADDLs can be used *inter alia* as a research tool in the study of ADDL binding and interaction within cells and in a method of assaying ADDL activity. Similarly, ADDLs, and studies of ADDL formation, activity and modulation can be employed *in vivo*.

10 In particular, the compounds identified using the methods of the present invention can be used to treat any one of a number of diseases, disorders, or conditions that result in deficits in cognition or learning (i.e., due to a failure of memory), and/or deficits in memory itself. Such treatment or prevention can be effected by administering compounds that prevent formation and/or activity of the
15 ADDLs, or that modulate (i.e., increase or decrease the activity of, desirably as a consequence of impacting ADDLs) the cell agents with which the ADDLs interact (e.g., so-called "downstream" events). Such compounds having ability to impact ADDLs are referred to herein as "ADDL-modulating compounds". ADDL-modulating compounds not only can act in a negative fashion, but also, in some cases
20 preferably are employed to increase the formation and/or activity of the ADDLs.
--- Desirably, when employed *in vivo*, the method can be employed for protecting an animal against decreases in cognition, learning or memory due to the effects of the ADDL protein assembly. This method comprises administering a compound that blocks the formation or activity of the ADDLs. Similarly, to the extent that deficits in
25 cognition, learning and/or memory accrue due to ADDL formation and/or activity, such deficits can be reversed or restored once the activity (and/or formation) of ADDLs is blocked. The invention thus preferably provides a method for reversing (or restoring) in an animal decreases in cognition, learning or memory due to the effects

of an oligomeric structure according to the invention. This method preferably comprises blocking the formation or activity of the ADDLs. The invention thus also desirably provides a method for reversing in a nerve cell decreases in long-term potentiation due to the effects of a soluble non-fibrillar amyloid β oligomeric structure according to the invention (as well as protecting a nerve cell against decrease in long-term potentiation due to the effects of a soluble non-fibrillar amyloid β oligomeric structure), the method comprising contacting the cell with a compound that blocks the formation or activity of the oligomeric structure.

In particular, this method desirably can be applied in the treatment or prevention of a disease, disorder, or condition that manifests as a deficit in cognition, learning and/or memory and which is due to ADDL formation or activity, especially a disease, disorder, or condition selected from the group consisting of Alzheimer's disease, adult Down's syndrome (i.e., over the age of 40 years), and senile dementia.

Also, this method desirably can be applied in the treatment or prevention of early deleterious effects on cellular activity, cognition, learning, and memory that may be apparent prior to the development of the disease, disorder, or condition itself, and which deleterious effects may contribute to the development of, or ultimately constitute the disease, disorder, or condition itself. In particular, the method preferably can be applied in the treatment or prevention of the early malfunction of nerve cells or other brain cells that can result as a consequence of ADDL formation or activity. Similarly, the method preferably can be applied in the treatment or prevention of focal memory deficits (FMD) such as have been described in the literature (e.g., Linn et al., *Arch. Neurol.*, 52, 485-490, 1995), in the event such FMD are due to ADDL formation or activity. The method further desirably can be employed in the treatment or prevention of ADDL-induced aberrant neuronal signaling, impairment of higher order writing skills (e.g., Snowden et al., *JAMA*, 275, 528-532, 1996) or other higher order cognitive function, decreases in (or absence of) long-term potentiation, that follows as a consequence of ADDL formation or activity.

According to this invention, "ADDL-induced aberrant neuronal signaling" can be measured by a variety of means. For instance, for normal neuronal signaling (as well as observation of a long-term potentiation response), it appears that among other things, Fyn kinase must be activated, Fyn kinase must phosphorylate the NMDA channel (Miyakawa et al., *Science*, 278, 698-701, 1997; Grant, *J Physiol Paris*, 90, 337-338, 1996), and Fyn must be present in the appropriate cellular location (which can be impeded by Fyn-FAK complex formation, for instance, as occurs in certain cytoskeletal reorganizations induced by ADDL). Based on this, ADDL-induced aberrant neuronal signaling (which is a signaling malfunction that is induced by aberrant activation of cellular pathways by ADDLs) and knowledge thereof can be employed in the methods of the invention, such as would be obvious to one skilled in the art. For instance, ADDL-induced aberrant cell signaling can be assessed (e.g., as a consequence of contacting nerve cells with ADDLs, which may further be conducted in the presence or absence of compounds being tested for ADDL-modulating activity) using any of these measures, or such as would be apparent to one skilled in the art, e.g., Fyn kinase activation (or alteration thereof), Fyn-FAK complex formation (or alteration thereof), cytoskeletal reorganization (or alteration thereof), Fyn kinase subcellular localization (or alteration thereof), Fyn kinase phosphorylation of the NMDA channel (or alteration thereof).

Furthermore, instead of using compounds that are identified using the methods of the invention, compounds known to have particular *in vitro* and *in vivo* effects can be employed to impact ADDLs in the above-described methods of treatment. Namely, amyloid formation can be (but need not necessarily be) modeled as a two-phase process. In the first phase is initiated the production of amyloid precursor protein (e.g., the amyloid precursor protein of 695 amino acids (Kang et al., *Nature*, 325, 733-736 (1987)) or the 751 amino acid protein (Ponte et al., *Nature*, 331, 525-527 (1988) each having within their sequence the β amyloid core protein sequence of approximately 4 kDa identified by Glenner et al. (U.S. Patent 4,666,829)). In the

second phase occurs amyloid processing and/or deposition into higher molecular weight structures (e.g., fibrils, or any other structure of β amyloid having a molecular weight greater than β amyloid monomer, and including structures that are considerably smaller than plaques and pre-plaques). It is conceivable that some compounds may impact one or both of these phases. For some compounds, a deleterious effect is obtained, but it is not clear whether the locus of inhibition is on protein production, or on amyloid processing and/or deposition.

Thus, relevant to this invention are compounds that act at either the first or second phase, or both phases. In particular, compounds that modulate the second phase have special utility to impact ADDLs and find use in methods of treatment that rely on ADDL modulation. Such compounds that modulate (e.g., block) the deposition of amyloid into higher molecular weight structures include, but are not limited to, compounds that modulate (particularly compounds that impede) the incorporation of β amyloid monomers into higher molecular weight structures, especially fibrils. Accordingly, desirably according to the invention, such compounds that impair incorporation of β amyloid monomers into higher molecular weight structures, particularly compounds that are known to inhibit fibril formation (and thus have been confirmed to inhibit incorporation of β amyloid into higher molecular weight structures), can be employed to exert an inhibitory effect on ADDL formation and/or activity (i.e., by reducing formation of ADDLs), in accordance with the methods of the invention. Of course, it is preferable that prior to such use, the ability of the modulators to impact ADDLs is confirmed, e.g., using the methods of the invention. Such known modulators that desirably can be employed in the present invention are described as follows, however, other similar modulators also can be employed.

In terms of compounds that act at the second phase, PCT International Application WO 96/39834 and Canadian Application 2222690 pertain to novel peptides capable of interacting with a hydrophobic structural determinant on a protein

or peptide for amyloid or amyloid-like deposit formation, thereby inhibiting and structurally blocking the abnormal folding of proteins and peptides into amyloid and amyloid-like deposits. In particular, the '834 application pertains to inhibitory peptides comprising a sequence of from about 3 to about 15 amino acid residues and
5 having a hydrophobic cluster of at least three amino acids, wherein at least one of the residues is a β -sheet blocking amino acid residue selected from Pro, Gly, Asn, and His, and the inhibitory peptide is capable of associating with a structural determinant on the protein or peptide to structurally block and inhibit the abnormal folding into amyloid or amyloid-like deposits.

10 PCT International Application WO 95/09838 pertains to a series of peptidergic compounds and their administration to patients to prevent abnormal deposition of β amyloid peptide.

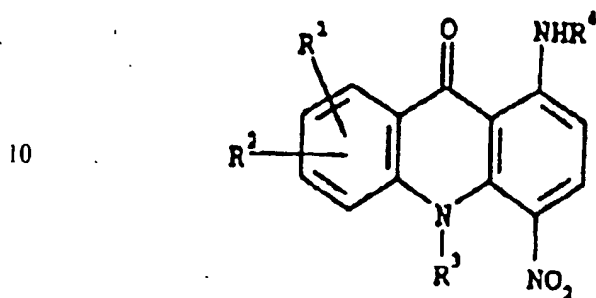
PCT International Application WO 98/08868 pertains to peptides that modulate natural β amyloid peptide aggregation. These peptide modulators comprise
15 three to five D-amino acid residues and include at least two D-amino acid residues selected from the group consisting of D-leucine, D-phenylalanine, and D-valine.

Similarly, PCT International Application WO 96/28471 pertains to an amyloid modulator compound that comprises an amyloidogenic protein or peptide fragment thereof (e.g., transthyretin, prion protein, islet amyloid polypeptide, atrial natriuretic
20 factor, kappa light chain, lambda light chain, amyloid A, procalcitonin, cystatin C, β 2-microglobulin, ApoA-1, gelsolin, procalcitonin, calcitonin, fibrinogen, and lysozyme) coupled directly or indirectly to at least one modifying group (e.g., comprises a cyclic, heterocyclic, or polycyclic group, contains a cis-decalin group, contains a cholanyl structure, is a cholyl group, comprises a biotin-containing group, a
25 fluorescein-containing group, etc.) such that the compound modulates the aggregation of natural amyloid proteins or peptides when contacted with these natural amyloidogenic proteins or peptides.

Also, PCT International Application WO 97/21728 pertains to peptides that

incorporate the Lys-Leu-Val-Phe-Phe (KVLFF) sequence of amyloid β that is necessary for polymerization to occur. Peptides that incorporate this sequence bind to amyloid β and are capable of blocking fibril formation.

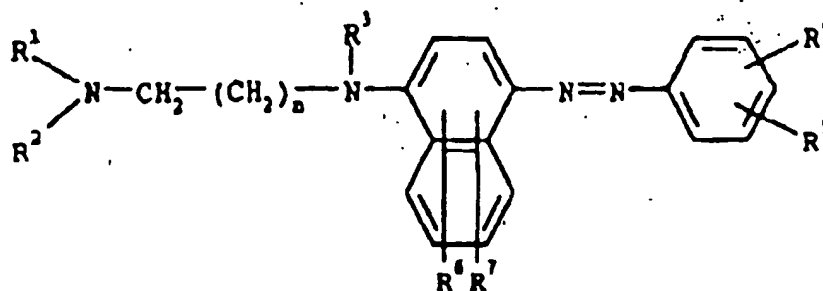
In terms of non-peptide agents, PCT International Application WO 97/16191
5 pertains to an agent for inhibiting the aggregation of amyloid protein in animals by administering a 9-acridinone compound having the formula



wherein R¹ and R² are hydrogen, halo, nitro, amino, hydroxy, trifluoromethyl, alkyl,
15 alkoxy, and alkythio; R³ is hydrogen or alkyl; and R⁴ is alkylene-N R⁵ R⁶, wherein R⁵
and R⁶ are independently hydrogen, C₁-C₄ alkyl, or taken together with the nitrogen to
which they are attached are piperidyl or pyrrolidinyl, and the pharmaceutically
acceptable salts thereof. The disclosed compounds previously were identified as
antibacterial and antitumor agents (U.S. Patent 4,626,540) and as antitumor agents
20 (Cholody et al., *J. Med. Chem.*, **33**, 49-52 (1990); Cholody et al., *J. Med. Chem.*, **35**,
378-382 (1992)).

PCT International Application WO 97/16194 pertains to an agent for
inhibiting the aggregation of amyloid protein in animals by administering a
naphthylazo compound having the formula

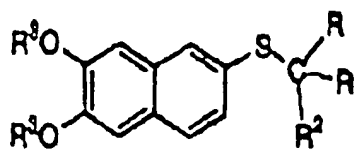
25



- 5 wherein R^1 and R^2 independently are hydrogen, alkyl, substituted alkyl, or a complete heterocyclic ring, R^3 is hydrogen or alkyl, R^4 , R^5 , R^6 , and R^7 are substituent groups including, but not limited to hydrogen, halo, alkyl, and alkoxy.

Japanese Patent 9095444 pertains to an agent for inhibiting the agglomeration and/or deposition of amyloid protein wherein this agent contains a thionaphthalene

- 10 derivative of the formula



15

- wherein R is a 1-5 carbon alkyl substituted with OH or COOR^4 (optionally substituted by aryl, heterocyclyl, COR^5 , CONHR^6 , or cyano; R^4 is H or 1-10 carbon alkyl, 3-10 carbon alkenyl, 3-10 carbon cyclic alkyl (all optionally substituted); R^5 and R^6 are
20 optionally substituted aryl or heterocyclyl; R^1 and R^2 are H , 1-5 carbon alkyl or phenyl; R^3 is hydrogen, 1-5 carbon alkyl or COR^7 ; R^7 is OR^8 , $-\text{R}''$ or $-\text{N}(\text{R}''')$; R^8 , R'' , R''' is 1-4 carbon alkyl.

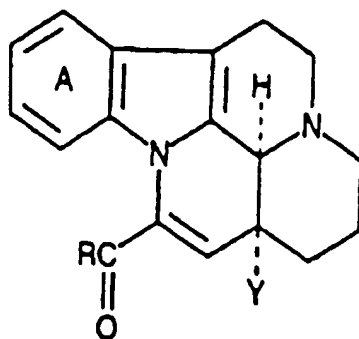
- Japanese Patent 7309760 and PCT International Application WO 95/11248
pertain to inhibitors of coagulation and/or deposition of amyloid β protein which are
25 particular rifamycin derivatives. Japanese Patent 7309759 pertains to inhibitors of coagulation and/or deposition of amyloid β protein which are particular rifamycin SV derivatives. Japanese Patent 7304675 pertains to inhibitors of agglutination and/or precipitation of amyloid β protein which are particular 3-homopiperazinyl-rifamycin

derivatives.

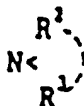
Japanese Patent 7247214 pertains to pyridine derivatives and that salts or prodrugs that can be employed as inhibitors of β -amyloid formation or deposition.

U.S. Patent 5,427,931 pertains to a method for inhibiting deposition of
5 amyloid plaques in a mammal that comprises the administration to the mammal of an effective plaque-deposition inhibiting amount of protease nexin-2, or a fragment or analog thereof.

In terms of compounds that may act at either the first or second phase (i.e., locus of action is undefined), PCT International Application WO 96/25161 pertains to
10 a pharmaceutical composition for inhibiting production or secretion of amyloid β protein, which comprises a compound having the formula



5 wherein ring A is an optionally substituted benzene ring, R represents OR^1 ,



or SR^1 , wherein R^1 , R^2 and R^3 are the same or different and each is selected from a hydrogen atom, an optionally substituted hydrocarbon group or R^2 and R^3 , taken
 10 together with the adjacent nitrogen atom, form an optionally substituted nitrogen-containing heterocyclic group, and Y is an optionally substituted alkyl group, or a pharmaceutically acceptable salt thereof, if necessary, with a pharmaceutically acceptable excipient, carrier or diluent. Of course, it is preferred that these and other known modulators (e.g., of the first phase or the second phase) are employed
 15 according to the invention. It also is preferred that gossypol and gossypol derivatives be employed. Furthermore, it is contemplated that modulators are employed that have ability to impact ADDL activity (e.g., PCT International Applications WO 93/15112 and 97/26913).

Also, the ADDLs themselves may be applied in treatment. It has been
 20 discovered that these novel assemblies described herein have numerous unexpected effects on cells that conceivably can be applied for therapy. For instance, ADDLs activate endothelial cells, which endothelial cells are known, among other things to interact with vascular cells. Along these lines, ADDLs could be employed, for instance, in wound healing. Also, by way of example, Botulinum Toxin Type A
 25 (BoTox) is a neuromuscular junction blocking agent produced by the bacterium

Clostridium botulinum that acts by blocking the release of the neurotransmitter acetylcholine. Botox has proven beneficial in the treatment of disabling muscle spasms, including dystonia. ADDLs themselves theoretically could be applied to either command neural cell function or, to selectively destroy targeted neural cells
5 (e.g., in cases of cancer, for instance of the central nervous system, particularly brain).
ADDLs appear further advantageous in this regard given that they have very early effects on cells, and given that their effect on cells (apart from their cell killing effect) appears to be reversible.

As discussed above, the ADDL-modulating compounds of the present
10 invention, compounds known to impact incorporation of amyloid β into higher molecular weight structures, as well as ADDLs themselves, can be employed to contact cells either *in vitro* or *in vivo*. According to the invention, a cell can be any cell, and, preferably, is a eukaryotic cell. A eukaryotic cell is a cell typically that possesses at some stage of its life a nucleus surrounded by a nuclear membrane.
15 Preferably the eukaryotic cell is of a multicellular species (e.g., as opposed to a unicellular yeast cell), and, even more preferably, is a mammalian (optionally human) cell. However, the method also can be effectively carried out using a wide variety of different cell types such as avian cells, and mammalian cells including but not limited to rodent, primate (such as chimpanzee, monkey, ape, gorilla, orangutan, or gibbon),
20 feline, canine, ungulate (such as ruminant or swine), as well as, in particular, human cells. Preferred cell types are cells formed in the brain, including neural cells and glial cells. An especially preferred cell type according to the invention is a neural cell (either normal or aberrant, e.g., transformed or cancerous). When employed in tissue culture, desirably the neural cell is a neuroblastoma cell.
25 A cell can be present as a single entity, or can be part of a larger collection of cells. Such a "larger collection of cells" can comprise, for instance, a cell culture (either mixed or pure), a tissue (e.g., neural or other tissue), an organ (e.g., brain or other organs), an organ system (e.g., nervous system or other organ system), or an

organism (e.g., mammal, or the like). Preferably, the organs/tissues/cells of interest in the context of the invention are of the central nervous system (e.g., are neural cells).

Also, according to the invention "contacting" comprises any means by which these agents physically touch a cell. The method is not dependent on any particular
5 means of introduction and is not to be so construed. Means of introduction are well known to those skilled in the art, and also are exemplified herein. Accordingly, introduction can be effected, for instance, either *in vitro* (e.g., in an *ex vivo* type method of therapy or in tissue culture studies) or *in vivo*. Other methods also are available and are known to those skilled in the art.

10 Such "contacting" can be done by any means known to those skilled in the art, and described herein, by which the apparent touching or mutual tangency of the ADDLs and ADDL-modulating compounds and the cell can be effected. For instance, contacting can be done by mixing these elements in a small volume of the same solution. Optionally, the elements further can be covalently joined, e.g., by
15 chemical means known to those skilled in the art, or other means, or preferably can be linked by means of noncovalent interactions (e.g., ionic bonds, hydrogen bonds, Van der Waals forces, and/or nonpolar interactions). In comparison, the cell to be affected and the ADDL or ADDL-modulating compound need not necessarily be brought into contact in a small volume; as, for instance, in cases where the ADDL or ADDL-
20 modulating compound is administered to a host, and the complex travels by the bloodstream or other body fluid such as cerebrospinal fluid to the cell with which it binds. The contacting of the cell with a ADDL or ADDL-modulating compound sometimes is done either before, along with, or after another compound of interest is administered. Desirably this contacting is done such that there is at least some
25 amount of time wherein the coadministered agents concurrently exert their effects on a cell or on the ADDL.

One skilled in the art will appreciate that suitable methods of administering an agent (e.g., an ADDL or ADDL-modulating compound) of the present invention to an

animal for purposes of therapy and/or diagnosis, research or study are available, and, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable excipients also are well-known to those who are skilled
5 in the art, and are readily available. The choice of excipient will be determined in part by the particular method used to administer the agent. Accordingly, there is a wide variety of suitable formulations for use in the context of the present invention. The following methods and excipients are merely exemplary and are in no way limiting.

Formulations suitable for oral administration can consist of (a) liquid
10 solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose,
15 acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient
20 in an inert base, such as gelatin and glycerin, emulsions, gels, and the like containing, in addition to the active ingredient, such excipients as are known in the art.

An agent of the present invention, alone or in combination with other suitable ingredients, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such
25 as dichlorodifluoromethane, propane, nitrogen, and the like. They also can be formulated as pharmaceuticals for non-pressured preparations such as in a nebulizer or an atomizer.

Formulations suitable for parenteral administration are preferred according to

the invention and include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

The dose administered to an animal, particularly a human, in the context of the present invention will vary with the agent of interest, the composition employed, the method of administration, and the particular site and organism being treated. However, preferably a dose corresponding to an effective amount of an agent (e.g., an ADDL or ADDL-modulating compound according to the invention) is employed. An "effective amount" is one that is sufficient to produce the desired effect in a host, which can be monitored using several end-points known to those skilled in the art. Some examples of desired effects include, but are not limited to, an effect on learning, memory, LTP response, neurotoxicity, ADDL formation, ADDL cell surface protein (e.g., receptor) binding, antibody binding, cell morphological changes, Fyn kinase activity, astrocyte activation, and changes in mRNA levels for proteins such as interleukin-1, inducible nitric oxide synthase, ApoE, ApoJ, and α 1-antichymotrypsin. These methods described are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan.

Moreover, with particular applications (e.g., either *in vitro* or *in vivo*) the actual dose and schedule of administration of ADDLs or ADDL-modulating compounds can vary depending on whether the composition is administered in combination with other pharmaceutical compositions, or depending on interindividual

differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts can vary in *in vitro* applications depending on the particular cell type utilized or the means or solution by which the ADDL or ADDL-modulating compound is transferred to culture. One skilled in the art easily can make any necessary

5 adjustments in accordance with the requirements of the particular situation.

With use of certain compounds, it may be desirable or even necessary to introduce the compounds (i.e., agents) as pharmaceutical compositions directly or indirectly into the brain. Direct techniques include, but are not limited to, the placement of a drug delivery catheter into the ventricular system of the host, thereby

10 bypassing the blood-brain barrier. Indirect techniques include, but are not limited to, the formulation of the compositions to convert hydrophilic drugs into lipid-soluble drugs using techniques known in the art (e.g., by blocking the hydroxyl, carboxyl, and primary amine groups present on the drug) which render the drug able to cross the blood-brain barrier. Furthermore, the delivery of hydrophilic drugs can be improved,

15 for instance, by intra-arterial infusion of hypertonic solutions (or other solutions) which transiently open the blood brain barrier.

Examples

The foregoing descriptions (as well as those which follow) are exemplary

20 only. Other applications of the method and constituents of the present invention will be apparent to one skilled in the art. Thus, the following examples further illustrate the present invention but, of course, should not be construed as in any way limiting the scope.

25 Example 1: Preparation of Amyloid β Oligomers

According to the invention, ADDLs were prepared by dissolving 1 mg of solid amyloid β 1-42 (e.g., synthesized as described in Lambert et al., *J. Neurosci. Res.*, 39, 377-395, 1994) in 44 μ L of anhydrous DMSO. This 5 mM solution then was diluted into cold (4°C) F12 media (Gibco BRL, Life Technologies) to a total volume of 2.20

mL (50-fold dilution), and vortexed for about 30 seconds. The mixture was allowed to incubate at from about 0°C to about 8°C for about 24 hours, followed by centrifugation at 14,000g for about 10 minutes at about 4°C. The supernatant was diluted by factors of 1:10 to 1:10,000 into the particular defined medium, prior to incubation with brain slice cultures, cell cultures or binding protein preparations. In general, however, ADDLs were formed at a concentration of A β protein of 100 μ M. Typically, the highest concentration used for experiments is 10 μ M and, in some cases, ADDLs (measured as initial A β concentration) were diluted (e.g., in cell culture media) to 1 nM. For analysis by atomic force microscopy (AFM), a 20 μ L aliquot of the 1:100 dilution was applied to the surface of a freshly cleaved mica disk and analyzed. Other manipulations were as described as follows, or as is apparent.

Alternately, ADDL formation was carried out as described above, with the exception that the F12 media was replaced by a buffer (i.e., "substitute F12 media") containing the following components: N, N-dimethylglycine (766 mg/L), D-glucose (1.802 g/L), calcium chloride (33 mg/L), copper sulfate pentahydrate (25 mg/L), iron(II) sulfate heptahydrate (0.8 mg/L), potassium chloride (223 mg/L), magnesium chloride (57 mg/L), sodium chloride (7.6 g/L), sodium bicarbonate (1.18 g/L), disodium hydrogen phosphate (142 mg/L), and zinc sulfate heptahydrate (0.9 mg/L). The pH of the buffer was adjusted to 8.0 using 0.1 M sodium hydroxide.

20

Example 2: Crosslinking of Amyloid β Oligomers

Glutaraldehyde has been successfully used in a variety of biochemical systems. Glutaraldehyde tends to crosslink proteins that are directly in contact, as opposed to nonspecific reaction with high concentrations of monomeric protein. In this example, glutaraldehyde-commanded crosslinking of amyloid β was investigated.

25

Oligomer preparation was carried out as described in example 1, with use of substitute F12 media. The supernatant that was obtained following centrifugation (and in some cases, fractionation) was treated with 0.22 mL of a 25% aqueous solution of

glutaraldehyde (Aldrich), followed by 0.67 mL of 0.175 M sodium borohydride in 0.1 M NaOH (according to the method of Levine, *Neurobiology of Aging*, 1995). The mixture was stirred at 4°C for 15 minutes and was quenched by addition of 1.67 mL of 20% aqueous sucrose. The mixture was concentrated 5 fold on a SpeedVac and dialyzed to remove components smaller than 1 kD. The material was analyzed by SDS PAGE. Gel filtration chromatography was carried according to the following: Superose 75PC 3.2/3.0 column (Pharmacia) was equilibrated with filtered and degassed 0.15% ammonium hydrogen carbonate buffer (pH=7.8) at a flow rate of 0.02 mL/min over the course of 18 h at room temperature. The flow rate was changed to 0.04 mL/min and 20 mL of solvent was eluted. 50 microliters of reaction solution was loaded on to the column and the flow rate was resumed at 0.04 mL/min. Compound elution was monitored via UV detection at 220 nm, and 0.5-1.0 mL fractions were collected during the course of the chromatography. Fraction No. 3, corresponding to the third peak of UV absorbance was isolated and demonstrated by AFM to contain globules 4.9 +/- 0.8 nm (by width analysis). This fraction was highly neurotoxic when contacted with brain slice neurons, as described in the examples which follow.

Example 3: Size Characterization of ADDLs

This example sets forth the size characterization of ADDLs formed as in Example 1, and using a variety of methods (e.g., native gel electrophoresis, SDS-polyacrylamide gel electrophoresis, AFM, field flow fractionation, and immunorecognition).

AFM was carried out essentially as described previously (e.g., Stine et al., *J. Protein Chem.*, 15, 193-203, 1996). Namely, images were obtained using a Digital Instruments (Santa Barbara, CA) Nanoscope IIIa Multimode Atomic force microscope using a J-scanner with xy range of 150µ. Tapping Mode was employed for all images using etched silicon TESP Nanoprobes (Digital Instruments). AFM data is analyzed

using the Nanoscope IIIa software and the IGOR Pro™ waveform analysis software. For AFM analysis, 4 μ scans (i.e., assessment of a 4 μ m x 4 μ m square) were conducted. Dimensions reported herein were obtained by section analysis, and where width analysis was employed, it is specified as being a value obtained by width
5 analysis. Section and width analysis are in separate analysis modules in the Nanoscope IIIa software. Generally, for ADDL analysis, there is a systematic deviation between the sizes obtained by section analysis and those obtained by width analysis. Namely, for a 4 μ scan, section analysis yields heights that are usually about 0.5 nm taller, thus resulting in a deviation of about 0.5 nm in the values obtained for
10 the sizes of the globules.

Analysis by gel electrophoresis was carried out on 15% polyacrylamide gels and visualized by Coomassie blue staining. ADDLs were resolved on 4-20% tris-glycine gels under non-denaturing conditions (Novex). Electrophoresis was performed at 20 mA for approximately 1.5 hours. Proteins were resolved with SDS-PAGE as
15 described in Zhang et al., *J. Biol. Chem.*, 269, 25247-25250, 1994. Protein was then visualized using silver stain (e.g. as described in Sherchenko et al., *Anal. Chem.*, 68, 850-858, 1996). Gel proteins from both native and SDS gels were transferred to nitrocellulose membranes according to Zhang et al. (*J. Biol. Chem.*, 269, 25247-50, 1994). Immunoblots were performed with biotinylated 6E10 antibody (Senetack, Inc.,
20 St. Louis, Missouri) at 1:5000 and visualized using ECL (Amersham). Typically, gels were scanned using a densitometer. This allowed provision of the computer-generated images of the gels (e.g., versus photographs of the gels themselves).

Size characterization of ADDLs by AFM section analysis (e.g., as described in Stine et al., *J. Protein Chem.*, 15, 193-203, 1996) or width analysis (Nanoscope III
25 software) indicated that the predominant species were globules of about 4.7 nm to about 6.2 nm along the z-axis. Comparison with small globular proteins ($\text{A}\beta$ 1-40 monomer, aprotinin, bFGF, carbonic anhydrase) suggested that ADDLs had mass between 17-42 kD. What appear to be distinct species can be recognized. These

appear to correspond to globules of dimensions of from about 4.9 nm to about 5.4 nm, from about 5.4 nm to about 5.7 nm, and from about 5.7 nm to about 6.2 nm. The globules of dimensions of about 4.9-5.4 nm and 5.7-6.2 nm appear to comprise about 50% of globules.

- 5 In harmony with the AFM analysis, SDS-PAGE immunoblots of ADDLs identified A β oligomers of about 17 kD to about 22 kD, with abundant 4 kD monomer present, presumably a breakdown product. Consistent with this interpretation, non-denaturing polyacrylamide gels of ADDLs show scant monomer, with a primary band near 30 kD, a less abundant band at ~17 kD, and no evidence of fibrils or aggregates.
- 10 Computer-generated images of a silver stained native gel and a Coomassie stained SDS-polyacrylamide gel are set out in Figure 1 and Figure 2, respectively. The correspondence between the SDS and non-denaturing gels confirms that the small oligomeric size of ADDLs was not due to detergent action. Oligomers seen in ADDL preparations were smaller than clusterin (Mr 80 kD, 40 kD in denatured gels), as
- 15 expected from use of low clusterin concentrations (1/40 relative to A β , which precluded association of A β as 1:1 A β -clusterin complexes).

 An ADDL preparation according to the invention was fractionated on a Superdex 75 column (Pharmacia, Superose 75PC 3.2/3.0 column). The fraction comprising the ADDLs was the third fraction of UV absorbance eluting from the

20 column and was analyzed by AFM and SDS-polyacrylamide gel electrophoresis. A representative AFM analysis of fraction 3 is depicted in Figure 3. Fractionation resulted in greater homogeneity for the ADDLs, with the majority of the globules having dimensions of from about 4.9 nm to about 5.4 nm. SDS-polyacrylamide gel electrophoresis of the fraction demonstrated a heavy lower band corresponding to

25 the monomer/dimer form of A β . As also observed for the non-fractionated preparation of ADDLs, this appears to be a breakdown product of the ADDLs. Heavier loading of the fraction revealed a larger-size broad band (perhaps a doublet). This further confirms the stability of the non-fibrillar oligomeric A β

structures to SDS.

Example 4: Clusterin Treatment of Amyloid β

5 Although it has been proposed that fibrillar structures represent the toxic form of A β (Lorenzo et al., *Proc. Natl. Acad. Sci. USA*, 91, 12243-12247, 1994; Howlett et al., *Neurodegen.*, 4, 23-32, 1995), novel neurotoxins that do not behave as sedimentable fibrils will form when A β 1-42 is incubated with low doses of clusterin, which also is known as "Apo J" (Oda et al., *Exper. Neurol.*, 136, 22-31, 1995; Oda et
10 al., *Biochem. Biophys. Res. Commun.*, 204, 1131-1136, 1994). To test if these slowly sedimenting toxins might still contain small or nascent fibrils, clusterin-treated A β preparations were examined by atomic force microscopy.

Clusterin treatment was carried out as described in Oda et al. (*Exper. Neurol.*, 136, 22-31, 1995) basically by adding clusterin in the incubation described in
15 Example 1. Alternatively, the starting A β 1-42 could be dissolved in 0.1 N HCl, rather than DMSO, and this starting A β 1-42 could even have fibrillar structures at the outset. However, incubation with clusterin for 24 hours at room temperature of 37 °C resulted in preparations that were predominantly free of fibrils, consistent with their slow sedimentation. This was confirmed by experiments showing that fibril
20 formation decreases as the amount of clusterin added increases.

The preparations resulting from clusterin treatment exclusively comprised small globular structures approximately 5-6 nm in size as determined by AFM analysis of ADDLs fractionated on a Superdex 75 gel column. Equivalent results were obtained by conventional electron microscopy. In contrast, A β 1-42 that had
25 self-associated under standard conditions (Snyder et al., *Biophys. J.*, 67, 1216-28, 1994) in the absence of clusterin showed primarily large, non-diffusible fibrillar species. Moreover, the resultant ADDL preparations were passed through a Centricon 10 kD cut-off membrane and analyzed on as SDS-polyacrylamide gradient gel. As can be seen in Figure 4, only the monomer passes through the Centricon 10 filter,

whereas ADDLs are retained by the filter. Monomer found after the separation could only be formed from the larger molecular weight species retained by the filter.

These results confirm that toxic ADDL preparations comprise small fibril-free oligomers of A β 1-42, and that ADDLs can be obtained by appropriate clusterin treatment of amyloid β .

Example 5: Physiologic Formation of ADDLs

10 The toxic moieties in Example 4 could comprise rare structures that contain oligomeric A β and clusterin. Whereas Oda et al. (*Exper. Neurol.*, 136, 22-31, 1995) reported that clusterin was found to increase the toxicity of A β 1-42 solutions, others have found that clusterin at stoichiometric levels protects against A β 1-40 toxicity (Boggs et al., *J. Neurochem.*, 67, 1324-1327, 1997). Accordingly, ADDL formation
15 in the absence of clusterin further was characterized in this Example.

When monomeric A β 1-42 solutions were maintained at low temperature in an appropriate media, formation of sedimentable A β fibrils was almost completely blocked. A β , however, did self-associate in these low-temperature solutions, forming ADDLs essentially indistinguishable from those chaperoned by clusterin. Finally,
20 ADDLs also formed when monomeric A β solutions were incubated at 37 degrees in brain slice culture medium but at very low concentration (50 nM), indicating a potential to form physiologically. All ADDL preparations were relatively stable and showed no conversion to fibrils during the 24-hour tissue culture experiments.

These results confirm that ADDLs form and are stable under physiological
25 conditions and suggest that they similarly can form and are stable *in vivo*.

Example 6: ADDLs are Diffusible, Extremely Potent CNS Neurotoxins

Whether ADDLs were induced by clusterin, low temperature, or low A β concentration, the stable oligomers that formed were potent neurotoxins. Toxicity
30 was examined in organotypic mouse brain slice cultures, which provided a

physiologically relevant model for mature CNS. Brain tissue was supported at the atmosphere-medium interface by a filter in order to maintain high viability in controls.

For these experiments, brain slices were obtained from strains B6 129 F2 and JR 2385 (Jackson Laboratories) and cultured as previously described (Stoppini et al.,
5 *J. Neurosci. Meth.*, 37, 173-182, 1991), with modifications. Namely, an adult mouse was sacrificed by carbon dioxide inhalation, followed by rapid decapitation. The head was emersed in cold, sterile dissection buffer (94 mL Gey's balanced salt solution, pH 7.2, supplemented with 2 mL 0.5M MgCl₂, 2 ml 25% glucose, and 2 mL 1.0 M Hepes), after which the brain was removed and placed on a sterile Sylgard-coated
10 plate. The cerebellum was removed and a mid-line cut was made to separate the cerebral hemispheres. Each hemisphere was sliced separately. The hemisphere was placed with the mid-line cut down and a 30 degree slice from the dorsal side was made to orient the hemisphere. The hemisphere was glued cut side down on the plastic stage of a Campden tissue chopper (previously wiped with ethanol) and
15 emersed in ice cold sterile buffer. Slices of 200 µm thickness were made from a lateral to medial direction, collecting those in which the hippocampus was visible.

Each slice was transferred with the top end of a sterile pipette to a small petri dish containing Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum, 2% S/P/F (streptomycin, penicillin, and fungizone; Life Technologies
20 (Gibco, BRL), Gaithersburg, MD), observed with a microscope to verify the presence of the hippocampus, and placed on a Millicell-CM insert (Millipore) in a deep well tissue culture dish (Falcon, 6-well dish). Each well contained 1.0 mL of growth medium, and usually two slices were on each insert. Slices were placed in a incubator (6% CO₂, 100% humidity) overnight. Growth medium was removed and wells were
25 washed with 1.0 mL warm Hanks BSS (Gibco, BRL, Life Technologies). Defined medium (DMEM, N2 supplements, SPF, e.g., as described in Bottenstein et al., *Proc. Natl. Acad. Sci.*, 76, 514-517, 1979) containing the amyloid β oligomers, with or without inhibitor compounds, was added to each well and the incubation was

continued for 24 hours.

Cell death was measured using the LIVE/DEAD® assay kit (Molecular Probes, Eugene, OR). This is a dual-label fluorescence assay in which live cells are detected by the presence of an esterase that cleaves calcein-AM to calcein, resulting in a green fluorescence. Dead cells take up ethidium homodimer, which intercalates with DNA and has a red fluorescence. The assay was carried out according to the manufacturer's directions at 2 μ M ethidium homodimer and 4 μ M calcein. Images were obtained within 30 minutes using a Nikon Diaphot microscope equipped with epifluorescence. The MetaMorph image analysis system (Universal Imaging Corporation, Philadelphia, PA) was used to quantify the number and/or area of cells showing green or red fluorescence.

For these experiments, ADDLs were present for 24 hours at a maximal 5 μ M dose of total A β (i.e., total A β was never more than 5 μ M in any ADDL experiment). Cell death, as shown by "false yellow staining", was almost completely confined to the stratum pyramidale (CA 3-4) and dentate gyrus (DG) suggesting strongly that principal neurons of the hippocampus (pyramidal and granule cells, respectively) are the targets of ADDL-induced toxicity. Furthermore, glia viability is unaffected by a 24 hour ADDL treatment of primary rat brain glia, as determined by trypan blue exclusion and MTT assay (Finch et al., unpublished). Dentate gyrus (DG) and CA3 regions were particularly sensitive and showed ADDL-evoked cell death in every culture-obtained from animals aged P20 (weanlings) to P84 (young adult). Up to 40% of the cells in this region die following chronic exposure to ADDLs. The pattern of neuronal death was not identical to that observed for NMDA, which killed neurons in DG and CA1 but spared CA3.

Some cultures from hippocampal DG and CA3 regions of animals more than 20 days of age were treated with conventional preparations of fibrillar A β . Consistent with the non-diffusible nature of the fibrils, no cell death (yellow staining) was evident even at 20 μ M. The staining pattern for live cells in this culture verified that

the CA3/dentate gyrus region of the hippocampus was being examined. The extent of cell death observed after conventional A β treatment (i.e., fibrillar A β preparations) was indistinguishable from negative controls in which cultures were given medium, or medium with clusterin supplement. In typical controls, cell death was less than 5%.

- 5 In fact, high viability in controls could be found even in cultures maintained several days beyond a typical experiment, which confirms that cell survival was not compromised by standard culture conditions.

A dose-response experiment was carried out to determine the potency of ADDLs in evoking cell death. Image analysis was used to quantify dead cell and live cell staining in fields containing the DG/CA3 areas. Figure 5 illustrates the % dead cells verses ADDL concentration measured as initial amyloid β 1-42 concentration (nM). Because of the difficulties of quantifying brain slices, the results are not detailed enough to determine the EC50 with precision. However, as can be seen in Figure 5, even after 1000-fold dilution (~ 5 nM A β), ADDL-evoked cell death was more than 20%. Toxicity was observed even with 0.3 nM ADDLs. This contrasts with results obtained with conventionally aged A β , which is toxic to neurons in culture at about 20 to about 50 μ M. These data show that ADDLs are effective at doses approximately 1,000-10,000-fold smaller than those used in fibrillar A β experiments.

- 20 These data from hippocampal slices thus confirm the ultratoxic nature of ADDLs. Furthermore, because ADDLs had to pass through the culture-support filter to cause cell death, the results validate that ADDLs are diffusible, consistent with their small oligomeric size. Also, the methods set forth herein can be employed as an assay for ADDL-mediated changes in cell viability. In particular,
- 25 the assay can be carried out by coincubating or coadministering along with the ADDLs agents that potentially may increase or decrease ADDL formation and/or activity. Results obtained with such coincubation or coadministration can be compared to results obtained with inclusion of ADDLs alone.

Example 7: MTT Oxidative Stress Toxicity Assay - PC12 Cells

This example sets forth an assay that can be employed to detect an early toxicity change in response to amyloid β oligomers.

- 5 For these experiments, PC12 cells were passaged at 4×10^4 cells/well on a 96-well culture plate and grown for 24 hours in DMEM + 10% fetal calf serum + 1% S/P/F (streptomycin, penicillin, and fungizone). Plates were treated with 200 $\mu\text{g/mL}$ poly-l-lysine for 2 hours prior to cell plating to enhance cell adhesion. One set of six wells was left untreated and fed with fresh media, while another set of wells was
- 10 treated with the vehicle control (PBS containing 10% 0.01 N HCl, aged o/n at RT). Positive controls were treated with triton (1%) and Na Azide (0.1%) in normal growth media. Amyloid β oligomers prepared as described in Example 1, or obtained upon coincubation with clusterin, with and without inhibitor compounds present, were added to the cells for 24 hours. After the 24 hour incubation, MTT (0.5 mg/mL) was
- 15 added to the cells for 2.5 hours (11 μL of 5 mg/ml stock solubilized in PBS into 100 μL of media). Healthy cells reduce the MTT into a formazan blue colored product. After the incubation with MTT, the media was aspirated and 100 μL of 100% DMSO was added to lyse the cells and dissolve the blue crystals. The plate was incubated for 15 min at RT and read on a plate reader (ELISA) at 550 nm.
- 20 The results of one such experiment are depicted in Figure 6. As can be seen from this figure, control cells not exposed to ADDLs ("Cont."), cells exposed to clusterin alone ("Apo J"), and cells exposed to monomeric $\text{A}\beta$ (" $\text{A}\beta$ ") show no cell toxicity. By contrast, cells exposed to amyloid β coaggregated with clusterin and aged one day (" $\text{A}\beta$:Apo J") show a decrease in MTT reduction, evidencing an early
- 25 toxicity change. The lattermost amyloid preparations were confirmed by AFM to lack amyloid fibrils.

Results of this experiment thus confirm that that ADDL preparations obtained from coaggregation of $\text{A}\beta$ mediated by clusterin have enhanced toxicity.

Moreover, the results confirm that the PC 12 oxidative stress response can be employed as an assay to detect early cell changes due to ADDLs. The assay can be carried out by coincubating or coadministering along with the ADDLs agents that potentially may increase or decrease ADDL formation and/or activity. Results
5 obtained with such coincubation or coadministration can be compared to results obtained with inclusion of ADDLs alone.

Example 8: MTT Oxidative Stress Toxicity Assay - HN2 Cells

This example sets forth a further assay of ADDL-mediated cell changes.

10 Namely, the MTT oxidative stress toxicity assay presented in the preceding example can be carried out with HN2 cells instead of PC12 cells. Other appropriate cells similarly can be employed.

For this assay, HN2 cells were passaged at 4×10^4 cells/well on a 96-well culture plate and grown for 24 hours in DMEM + 10% fetal calf serum + 1% S/P/F
15 (streptomycin, penicillin, and fungizone). Plates were treated with 200 $\mu\text{g/mL}$ poly l-lysine for 2 hours prior to cell plating to enhance cell adhesion. The cells were differentiated for 24-48 hours with 5 μM retinoic acid and growth was further inhibited with 1% serum. One set of wells was left untreated and given fresh media. Another set of wells was treated with the vehicle control (0.2% DMSO). Positive
20 controls were treated with triton (1%) and Na Azide (0.1%). Amyloid β oligomers prepared as described in example 1, with and without inhibitor compounds present, were added to the cells for 24 hours. After the 24 hour incubation, MTT (0.5 mg/mL) was added to the cells for 2.5 hours (11 μL of 5 mg/mL stock into 100 μL of media). After the incubation with MTT, the media was aspirated and 100 μL of 100% DMSO
25 is added to lyse the cells and dissolve the blue crystals. The plate was incubated for 15 minutes at RT and read on a plate reader (ELISA) at 550 nm.

This assay similarly can be carried out by coincubating or coadministering along with the ADDLs agents that potentially may increase or decrease ADDL

formation and/or activity. Results obtained with such coincubation or coadministration can be compared to results obtained with inclusion of ADDLs alone.

5 **Example 9: Cell Morphology by Phase Microscopy**

This example sets forth yet another assay of ADDL-mediated cell changes – assay of cell morphology by phase microscopy.

For this assay, cultures were grown to low density (50-60% confluence). To initiate the experiment, the cells were serum-starved in F12 media for 1 hour. Cells
10 were then incubated for 3 hours with amyloid β oligomers prepared as described in example 1, with and without inhibitor compounds added to the cells, for 24 hours. After 3 hours, cells were examined for morphological differences or fixed for immunofluorescence labeling. Samples were examined using the MetaMorph Image Analysis system and an MRI video camera (Universal Imaging, Inc.).

15 Results of such assays are presented in the examples which follow. In particular, the assay can be carried out by coincubating or coadministering along with the ADDLs agents that potentially may increase or decrease ADDL formation and/or activity. Results obtained with such coincubation or coadministration can be compared to results obtained with inclusion of ADDLs alone.

20

Example 10: FACScan Assay for Binding of ADDLs to Cell Surfaces

Because cell surface receptors recently have been identified on glial cells for conventionally prepared A β (Yan et al., *Nature*, 382, 685-691, 1996; El Khoury et al., *Nature*, 382, 716-719, 1996), and because neuronal death at low ADDL doses
25 suggested possible involvement of signaling mechanisms, experiments were undertaken to determine if specific cell surface binding sites on neurons exist for ADDLs.

For flow cytometry, cells were dissociated with 0.1% trypsin and plated at least overnight onto tissue culture plastic at low density. Cells were removed with

cold phosphate buffered saline (PBS) /0.5 mM EDTA, washed three times and resuspended in ice-cold PBS to a final concentration of 500,000 cells/mL. Cells were incubated in cold PBS with amyloid β oligomers prepared as described in Example 1, except that 10% of the amyloid β is an amyloid β 1-42 analog containing biocytin at position 1 replacing aspartate. Oligomers with and without inhibitor compounds present were added to the cells for 24 hours. The cells were washed twice in cold PBS to remove free, unbound amyloid β oligomers, resuspended in a 1:1,000 dilution of avidin conjugated to fluorescein, and incubated for one hour at 4°C with gentle agitation. Alternately, amyloid β -specific antibodies and fluorescent secondary antibody were employed instead of avidin, eliminating the need to incorporate 10% of the biotinylated amyloid β analog. Namely, biotinylated 6E10 monoclonal antibody (1 μ L Senetec, Inc., St. Louis, Missouri) was added to the cell suspension and incubated for 30 minutes. Bound antibody was detected after pelleting cells and resuspending in 500 μ L PBS, using FITC-conjugated streptavidin (1:500, Jackson Laboratories) for 30 minutes.

Cells were analyzed by a Becton-Dickenson Fluorescence Activated Cell Scanner (FACScan). 10,000 or 20,000 events typically were collected for both forward scatter (size) and fluorescence intensity, and the data were analyzed by Consort 30 software (Becton-Dickinson). Binding was quantified by multiplying mean fluorescence by total number of events, and subtracting value for background cell fluorescence in the presence of 6E10 and FITC.

For these experiments, FACScan analysis was done to compare ADDL immunoreactivity in suspensions of log-phase yeast cells (a largely carbohydrate surface) and of the B103 CNS neuronal cell line (Schubert et al., *Nature*, 249, 224-227, 1974). For B103 cells, addition of ADDLs caused a major increase in cell associated fluorescence, as shown in Figure 7. Trypsin treatment of the B103 cells for 1 minute eliminated ADDL binding. In contrast, control yeast cells (data not shown) demonstrated no ADDL binding, verifying the selectivity of ADDLs for

proteins present on the cell surface. Suspensions of hippocampal cells (trypsinized tissue followed by a two-hour metabolic recovery) also bound ADDLs, but with a reduced number of binding events compared with the B103 cells, as evidenced by the reduced fluorescence intensity of the labelled peak. This appears in Figure 8 as a
5 leftward shifting of the labelled peak.

These results thus suggest that the ADDLs exert their effects by binding to a specific cell surface receptor. In particular, the trypsin sensitivity of B103 cells showed that their ADDL binding sites were cell surface proteins and that binding was selective for a subset of particular domains within these proteins.

10 Moreover, the present assay can also be employed as an assay for ADDL-mediated cell binding. In particular, the assay can be carried out by coincubating or coadministering along with the ADDLs agents that potentially may increase or decrease ADDL formation and/or activity. Results obtained with such coincubation or coadministration can be compared to results obtained with inclusion of ADDLs
15 alone.

Example 11: Inhibition of ADDL Formation by Gossypol

This example sets forth the manner in which ADDL formation can be inhibited using, for instance, gossypol.

For these experiments, ADDLs were prepared as described in Example 1.

20 Gossypol (Aldrich) was added to a concentration of 100 μ M during the incubation of the A β protein to form ADDLs. The resulting preparation was assessed for neurotoxicity using the LIVE/DEAD® assay kit as previously described. The amount of cell death that occurred after 24 hours of exposure to the gossypol/ADDL preparation was less than 5%. This is comparable to the level of toxicity obtained for
25 a corresponding DMSO control preparation (i.e., 6%), or a gossypol control preparation that did not contain any ADDLs (i.e., 4%).

These results thus confirm that compounds such as gossypol can be employed to inhibit ADDL formation.

Example 12: Inhibition of ADDL Binding by Tryptic Peptides

Because B103 cell trypsinization was found to block subsequent ADDL binding, experiments were done as set forth in this example to test if tryptic fragments released from the cell surface retard ADDL binding activity.

Tryptic peptides were prepared using confluent B103 cells from four 100 mm dishes that were removed by trypsinization (0.025%, Life Technologies) for approximately 3 minutes. Trypsin-chymotrypsin inhibitor (Sigma, 0.5 mg/mL in Hank's Buffered Saline) was added, and cells were removed via centrifugation at 500 x g for 5 minutes. Supernatant (~12 mL) was concentrated to approximately 1.0 mL using a Centricon 3 filter (Amicon), and was frozen after the protein concentration was determined. For blocking experiments, sterile concentrated tryptic peptides (0.25 mg/mL) were added to the organotypic brain slice or to the suspended B103 cells in the FACs assay at the same time as the ADDLs were added.

In FACScan assays, tryptic peptides released into the culture media (0.25 mg/mL) inhibited ADDL binding by > 90% as shown in Figure 9. By comparison, control cells exposed to BSA, even at 100mg/mL, had no loss of binding. Tryptic peptides, if added after ADDLs were already attached to cells, did not significantly lower fluorescence intensities. This indicates that the peptides did not compromise the ability of the assay to quantify bound ADDLs. Besides blocking ADDL binding, the tryptic peptides also were antagonists of ADDL-evoked cell death. Namely, as shown in Figure 9, addition of tryptic peptides resulted in a 75% reduction in cell death, $p < 0.002$.

These data confirm that particular cell surface proteins mediate ADDL binding, and that solubilized tryptic peptides from the cell surface provide neuroprotective, ADDL-neutralizing activity. Moreover, the present assay can also be employed as an assay for agents that mediate ADDL cell binding or ADDL effects on cell activity. In particular, the assay can be carried out by coincubating or

coadministering along with the ADDLs agents that potentially may increase or decrease ADDL formation and/or activity. Results obtained with such coincubation or coadministration can be compared to results obtained with inclusion of ADDLs alone. Moreover, addition of the agents before or after binding of the ADDLs to the cell surface can be compared to identify agents that impact such binding, or that act after binding has occurred.

Example 13: Dose Response Curve for ADDL Cell Binding

This example sets forth dose response experiments done to determine whether ADDL binding to the cell surface is saturable. Such saturability would be expected if the ADDLs in fact interact with a particular cell surface receptor.

For these studies, B103 cells were incubated with increasing amounts of ADDLs and ADDL binding was quantitated by FACScan analysis. Results are presented in Figure 10. These results confirm that a distinct plateau is achieved for ADDL binding. Saturability of ADDL binding occurs at a relative A β 1-42 concentration (i.e., ADDL concentration relative to A β) of about 250 nm.

These results thus confirm that ADDL binding is saturable. Such saturability of ADDL binding, especially when considered with the results of the trypsin studies, validates that the ADDLs are acting through a particular cell surface receptor.

Example 14: Cell-Based ELISA for ADDL Binding Activity

This example sets forth a cell-based assay, particularly a cell-based enzyme-linked immunosorbent assay (ELISA) that can be employed to assess ADDL binding activity.

For these studies, 48 hours prior to conduct of the experiment, 2.5×10^4 B103 cells present as a suspension in 100 μ L DMEM were placed in each assay well of a 96-well microtiter plate and kept in an incubator at 37°C. 24 hours prior to the conduct of the experiment, ADDLs were prepared according to the method described

in Example 1. To begin the assay, each microtiter plate well containing cells was treated with 50 μ L of fixative (3.7% formalin in DMEM) for 10 minutes at room temperature. This fixative/DMEM solution was removed and a second treatment with 50 μ L formalin (no DMEM) was carried out for 15 minutes at room temperature. The
5 fixative was removed and each well was washed twice with 100 μ L phosphate buffered saline (PBS). 200 μ L of a blocking agent (1% BSA in PBS) was added to each well and incubated at room temperature for 1 hour. After 2 washes with 100 μ L PBS, 50 μ L of ADDLs (previously diluted 1:10 in PBS), were added to the appropriate wells, or PBS alone as a control, and the resulting wells were incubated at
10 37°C for 1 hour. 3 washes with 100 μ L PBS were carried out, and 50 μ L biotinylated 6E10 (Senetek) diluted 1:1000 in 1% BSA/PBS was added to the appropriate wells. In other wells, PBS was added as a control. After incubation for 1 hour at room temperature on a rotator, the wells were washed 3 times with 50 μ L PBS, and 50 μ L of the ABC reagent (Elite ABC kit, Vector Labs) was added and incubated for 30
15 minutes at room temperature on the rotator. After washing 4 times with 50 μ L PBS, 50 μ L of ABTS substrate solution was added to each well and the plate was incubated in the dark at room temperature. The plate was analyzed for increasing absorption at 405 nm. Only when ADDLs, cells, and 6E10 were present was there a significant signal, as illustrated in Figure 11.

20 These results further confirm that a cell-based ELISA assay can be employed as an assay for ADDL-mediated cell binding. In particular, the assay can be carried out by coincubating or coadministering along with the ADDLs agents that potentially may increase or decrease ADDL formation and/or activity. Results obtained with such coincubation or coadministration can be compared to results obtained with
25 inclusion of ADDLs alone.

Example 15: Fyn kinase knockout protects against ADDL neurotoxicity

To investigate further the potential involvement of signal transduction in

ADDL toxicity, the experiments in this example compared the impact of ADDLs on brain slices from isogenic *fyn*^{-/-} and *fyn*^{+/+} animals. Fyn belongs to the Src-family of protein tyrosine kinases, which are central to multiple cellular signals and responses (Clarke et al., *Science*, 268, 233-238). Fyn is of particular interest because it is upregulated in AD-afflicted neurons (Shirazi et al., *Neuroreport*, 4, 435-437, 1993). It also appears to be activated by conventional A β preparations (Zhang et al., *Neurosci. Letts.*, 211, 187-190, 1996) which subsequently have been shown to contain ADDLs by AFM. Fyn knockout mice, moreover, have reduced apoptosis in the developing hippocampus (Grant et al., *Science*, 258, 1903-1910, 1992).

10 For these studies, Fyn knockout mice (Grant et al., *Science*, 258, 1903-1910, 1992) were treated as described in the preceding examples, by comparing images of brain slices of mice either treated or not treated with ADDLs for 24 hours to determine dead cells in the DG and CA3 area. The quantitative comparison (presented in Figure 12) was obtained with error bars representing means \pm SEM for 15 4-7 slices.

In contrast to cultures from wild-type animals, cultures from *fyn*^{-/-} animals showed negligible ADDL-evoked cell death, as shown in Figure 12. For ADDLs, the level of cell death in *fyn*^{+/+} slices was more than five times that in *fyn*^{-/-} cultures. In *fyn*^{-/-} cultures, cell death in the presence of ADDLs was at background level. The 20 neuroprotective response was selective; hippocampal cell death evoked by NMDA receptor agonists (Bruce et al., *Exper. Neurol.*, 132, 209-219, 1995; Vornov et al., *Neurochem.*, 56, 996-1006, 1991) was unaffected (not shown). Analysis (ANOVA) using the Tukey multiple comparison gave a value of $P < 0.001$ for the ADDL *fyn*^{+/+} data compared to all other conditions.

25 These results confirm that loss of Fyn kinase protected DG and CA3 hippocampal regions from cell death induced by ADDLs. The results validate that ADDL toxicity is mediated by a mechanism blocked by knockout of Fyn protein tyrosine kinase. These results further suggest that neuroprotective benefits can be

obtained by treatments that abrogate the activity of Fyn protein tyrosine kinase or the expression of the gene encoding Fyn protein kinase.

Example 16: Astrocyte Activation Experiments

5 To investigate further the potential involvement of signal transduction in ADDL toxicity, the experiments in this example compared the impact on ADDLs on activation of astrocytes.

For these experiments, cortical astrocyte cultures were prepared from neonatal (1-2 day old) Sprague-Dawley rat pups by the method of Levison and McCarthy
10 (Levison et al., in: Banker et al. (Eds.), *Culturing Nerve Cells*, MIT press, Cambridge, MA, , 309-36, 1991), as previously described (Hu et al., *J. Biol. Chem.*, 271, 2543-2547, 1996). Briefly, cerebral cortex was dissected out, trypsinized, and cells were cultured in α -MEM (Gibco, BRL) containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan UT) and antibiotics (100 U/mL penicillin, 100
15 mg/mL streptomycin). After 11 days in culture, cells were trypsinized and replated into 100-mm plates at a density of $\sim 6 \times 10^5$ cells/plate and grown until confluent (Hu et al., *J. Biol. Chem.*, 271, 2543-2547, 1996).

Astrocytes were treated with ADDLs prepared according to Example 1, or with A β 17-42 (synthesized as per Lambert et al., *J. Neurosci. Res.*, 39, 377-384,
20 1994; also commercially available). Treatment was done by trypsinizing confluent cultures of astrocytes and plating onto 60 mm tissue culture dishes at a density of 1×10^6 cells/dish (e.g., for RNA analysis and ELISAs), into 4-well chamber slides at 5×10^4 cells/well (e.g., for immunohistochemistry), or into 96-well plates at a density of 5×10^4 cells/well (e.g., for NO assays). After 24 hours of incubation, the cells were
25 washed twice with PBS to remove serum, and the cultures incubated in α -MEM containing N2 supplements for an additional 24 hours before addition of A β peptides or control buffer (i.e., buffer containing diluent).

Examination of astrocyte morphology was done by examining cells under a

Nikon TMS inverted microscope equipped with a Javelin SmartCam camera, Sony video monitor and color video printer. Typically, four arbitrarily selected microscopic fields (20X magnification) were photographed for each experimental condition. Morphological activation was quantified from the photographs with NIH Image by
5 counting the number of activated cells (defined as a cell with one or more processes at least one cell body in length) in the four fields.

The mRNA levels in the cultures was determined with use of Northern blots and slot blots. This was done by exposing cells to ADDLs or control buffer for 24 hours. After this time, the cells were washed twice with diethylpyrocarbonate
10 (DEPC)-treated PBS, and total RNA was isolated by RNeasy purification mini-columns (Qiagen, Inc., Chatsworth, CA), as recommended by the manufacturer. Typical yields of RNA were 8 to 30 mg of total RNA per dish. For Northern blot analysis, 5 mg total RNA per sample was separated on an agarose-formaldehyde gel, transferred by capillary action to Hybond-N membrane (Amersham, Arlington
15 Heights IL), and UV crosslinked. For slot blot analysis, 200 ng of total RNA per sample was blotted onto Duralon-UV membrane (Stratagene, La Jolla CA) under vacuum, and UV crosslinked. Confirmation of equivalent RNA loadings was done by ethidium bromide staining or by hybridization and normalization with a GAPDH probe.

20 Probes were generated by restriction enzyme digests of plasmids, and subsequent gel purification of the appropriate fragment. Namely, cDNA fragments were prepared by RT-PCR using total RNA from rat cortical astrocytes. RNA was reverse transcribed with a Superscript II system (GIBCO/ BRL), and PCR was performed on a PTC-100 thermal controller (MJ Research Inc, Watertown, MA) using
25 35 cycles at the following settings: 52°C for 40 seconds; 72°C for 40 seconds; 96°C for 40 seconds. Primer pairs used to amplify a 447 bp fragment of rat IL-1 β were: Forward: 5' GCACCTTCTTCCCTTCATC 3' [SEQ ID NO:1]. Reverse: 5' TGCTGATGTACCAGTTGGGG 3' [SEQ ID NO:2]. Primer pairs used to amplify a

435 bp fragment of rat GFAP were: Forward: 5' CAGTCCTTGACCTGCGACC 3' [SEQ ID NO:3]. Reverse: 5' GCCTCACATCACATCCTTG 3' [SEQ ID NO:4]. PCR products were cloned into the pCR2.1 vector with the Invitrogen TA cloning kit, and constructs were verified by DNA sequencing. Probes were prepared by *EcoRI* digestion of the vector, followed by gel purification of the appropriate fragments. The plasmids were the rat iNOS cDNA plasmid pAstNOS-4, corresponding to the rat iNOS cDNA bases 3007-3943 (Galea et al., *J. Neurosci. Res.*, 37, 406-414, 1994), and the rat GAPDH cDNA plasmid pTRI-GAPDH (Ambion, Inc., Austin TX).

The probes (25 ng) were labeled with ³²P-dCTP by using a Prime-a-Gene Random-Prime labeling kit (Promega, Madison WI) and separated from unincorporated nucleotides by use of push-columns (Stratagene). Hybridization was done under stringent conditions with QuikHyb solution (Stratagene), using the protocol recommended for stringent hybridization. Briefly, prehybridization was conducted at 68°C for about 30 to 60 minutes, and hybridization was at 68°C for about 60 minutes. Blots were then washed under stringent conditions and exposed to either autoradiography or phosphoimaging plate. Autoradiograms were scanned with a BioRad GS-670 laser scanner, and band density was quantified with Molecular Analyst v2.1 (BioRad, Hercules CA) image analysis software. Phosphoimages were captured on a Storm 840 system (Molecular Dynamics, Sunnyvale CA), and band density was quantified with Image Quant v1.1 (Molecular Dynamics) image analysis software.

For measurement of NO by nitrite assay, cells were incubated with A β peptides or control buffer for 48 hours, and then nitrite levels in the conditioned media were measured by the Griess reaction as previously described (Hu et al., *J. Biol. Chem.*, 271, 2543-2547, 1996). When the NOS inhibitor N-nitro-L-arginine methylester (L-name) or the inactive D-name isomer were used, these agents were added to the cultures at the same time as the A β .

Results of these experiments are presented in Figure 13. As can be seen in this

figure, glia activation increases when astrocytes are incubated with ADDLs, but not when astrocytes are incubated with A β 17-42.

These results confirm that ADDLs activate glial cells. It is possible that glial proteins may contribute to neural deficits, for instance, as occur in

5 Alzheimer's Disease, and that some effects of ADDLs may actually be mediated indirectly by activation of glial cells. In particular, glial proteins may facilitate formation of ADDLs, or ADDL-mediated effects that occur downstream of receptor binding. Also, it is known that clusterin is upregulated in the brain of the

10 Alzheimer's diseased subject, and clusterin is made at elevated levels only in glial cells that are activated. Based on this, activation of glial cells by a non-ADDL, non-amyloid stimulus could produce clusterin which in turn might lead to ADDLs, which in turn would damage neurons and cause further activation of glial cells.

Regardless of the mechanism, these results further suggest that neuroprotective benefits can be obtained by treatments that modulate (i.e., increase

15 or decrease) ADDL-mediated glial cell activation. Further, the results suggest that blocking these effects on glial cells, apart from blocking the neuronal effects, may be beneficial.

Example 17: LTP Assay—ADDLs Disrupt LTP

20 Long-term potentiation (LTP) is a classic paradigm for synaptic plasticity and a model for memory and learning, faculties that are selectively lost in early stage AD. This example sets forth experiments done to examine the effects of ADDLs on LTP, particularly medial perforant path-granule cell LTP.

Injections of intact animals: Mice were anesthetized with urethane and

25 placed in a stereotaxic apparatus. Body temperature was maintained using a heated water jacket pad. The brain surface was exposed through holes in the skull. Bregma and lambda positions for injection into the middle molecular layer of hippocampus are 2 mm posterior to bregma, 1 mm lateral to the midline, and 1.2-1.5 mm ventral to the

brain surface. Amyloid β oligomer injections were by nitrogen puff through ~ 10 nm diameter glass pipettes. Volumes of 20-50 nL of amyloid β oligomer solution (180 nM of amyloid β in phosphate buffered saline, PBS) were given over the course of an hour. Control mice received an equivalent volume of PBS alone. The animal was
5 allowed to rest for varying time periods before the LTP stimulus is given (typically 60 minutes).

LTP in injected animals: Experiments follow the paradigm established by Routtenberg and colleagues for LTP in mice (Namgung et al., *Brain Research*, 689, 85-92, 1995). Perforant path stimulation from the entorhinal cortex was used, with
10 recording from the middle molecular layer and the cell body of the dentate gyrus. A population excitatory postsynaptic potential (pop-EPSP) and a population spike potential (pop-spike) were observed upon electrical stimulation. LTP could be induced in these responses by a stimulus of 3 trains of 400 Hz, 8 x 0.4 ms pulses/train (Namgung et al., *Brain Research*, 689, 85-92, 1995). Recordings were taken for 2-3
15 hours after the stimulus (i.e., applied at time 0) to determine if LTP is retained. The animal was then sacrificed immediately, or was allowed to recover for either 1, 3, or 7 days and then sacrificed as above. The brain was cryoprotected with 30% sucrose, and then sectioned (30 μ M) with a microtome. Some sections were placed on slides subbed with gelatin and others were analyzed using a free-floating protocol.

20 Immunohistochemistry was used to monitor changes in GAP-43, in PKC subtypes, and in protein phosphorylation of tau (PHF-1), paxillin, and focal adhesion kinase. Wave forms were analyzed by machine as described previously (Colley et al., *J. Neurosci.*, 10, 3353-3360, 1990). A 2-way ANOVA compares changes in spike amplitude between treated and untreated groups.

25 **Figure 14** illustrates the spike amplitude effect of ADDLs in whole animals. As can be clearly seen in this figure, ADDLs block the persistence phase of LTP induced by high frequency electrical stimuli applied to entorhinal cortex and measured as cell body spike amplitude in middle molecular layer of the dentate

gyrus.

After the LTP experiment was performed, animals were allowed to recover for various times and then sacrificed using sodium pentobarbital anesthetic and perfusion with 4% paraformaldehyde. For viability studies, times of 3 hours, 24
5 hours, 3 days, and 7 days were used. The brain was cryoprotected with 30% sucrose and then sectioned (30 μ M) with a microtome. Sections were placed on slides subbed with gelatin and stained initially with cresyl violet. Cell loss was measured by counting cell bodies in the dentate gyrus, CA3, CA1, and entorhinal cortex, and correlated with dose and time of exposure of ADDLs. The results of
10 these experiments confirmed that no cell death occurred as of 24 hours following the LTP experiments.

Similarly, the LTP response was examined in hippocampal slices from young adult rats. As can be seen in Figure 15, incubation of rat hippocampal slices with ADDLs prevents LTP well before any overt signs of cell degeneration. Hippocampal
15 slices (n=6) exposed to 500 nM ADDLs for 45 minutes prior showed no potentiation in the population spike 30 minutes after the tetanic stimulation (mean amplitude 99% \pm 7.6), despite a continuing capacity for action potentials. In contrast, LTP was readily induced in slices incubated with vehicle (n=6), with an amplitude of 138% \pm 8.1 for the last 10 minutes; this value is comparable to that previously demonstrated in
20 this age group (Trommer et al., *Exper. Neurol.*, 131, 83-92, 1995). Although LTP was absent in ADDL-treated slices, their cells were competent to generate action potentials and showed no signs of degeneration.

These results validate that in both whole animals and tissue slices, the addition of ADDLs results in significant disruption of LTP in less than an hour, prior to any
25 cell degeneration or killing. These experiments thus support that ADDLs exert very early effects, and interference with ADDL formation and/or activity thus can be employed to obtain a therapeutic effect prior to advancement of a disease, disorder, or condition (e.g., Alzheimer's disease) to a stage where cell death results. In other

words, these results confirm that decreases in memory occur before neurons die. Interference prior to such cell death thus can be employed to reverse the progression, and potentially restore decreases in memory.

5 Example 18: Early Effects of ADDLs *in vivo*

This example sets forth early effects of ADDLs *in vivo* and the manner in knowledge of such early effects can be manipulated.

The primary symptoms of Alzheimer's disease involve learning and memory deficits. However, the link between behavioral deficits and aggregated amyloid
10 deposits has been difficult to establish. In transgenic mice, overexpressing mutant APP under the control of the platelet-derived growth factor promoter results in the deposition of large amounts of amyloid (Games et al., *Nature*, 373, 523-527, 1995). By contrast, no behavioral deficits have been reported using this system. Other researchers (i.e., Nalbantoglu et al., *Nature*, 387, 500-505, 1997 and Holcomb et al.,
15 *Nat. Med.*, 4, 97-100, 1998) working with transgenic mice report observing significant behavioral and cognitive deficits that occur well before any significant deposits of aggregated amyloid are observed. These behavioral and cognitive defects include failure to long-term potentiate (Nalbantoglu et al., *supra*). These models collectively suggest that non-deposited forms of amyloid are responsible for the early cognitive
20 and behavioral deficits that occur as a result of induced neuronal malfunction. It is consistent with these models that the novel ADDLs described herein are this non-deposited form of amyloid causing the early cognitive and behavioral defects. In view of this, ADDL modulating compounds according to the invention can be employed in the treatment and/or prevention of these early cognitive and behavioural deficits
25 resulting from ADDL-induced neuronal malfunction, or ADDLs themselves can be applied, for instance, in animal models, to study such induced neuronal malfunction.

Similarly, in elderly humans, cognitive decline and focal memory deficits can occur well before a diagnosis of probable stage I Alzheimer's disease is made (Linn et

al., *Arch. Neurol.*, 52, 485-490, 1995). These focal memory deficits may result from induced aberrant signaling in neurons, rather than cell death. Other functions, such as higher order writing skills (Snowdon et al., *JAMA*, 275, 528-532, 1996) also may be affected by aberrant neuronal function that occurs long before cell death. It is
5 consistent with what is known regarding these defects, and the information regarding ADDLs provided herein, that ADDLs induce these defects in a manner similar to compromised LTP function such as is induced by ADDLs. Along these lines, ADDL modulating compounds according to the invention can be employed in the treatment and/or prevention of these early cognitive decline and focal memory deficits, and
10 impairment of higher order writing skills, resulting from ADDL formation or activity, or ADDLs themselves can be applied, for instance, in animal models, to study such induced defects. In particular, such studies can be conducted such as is known to those skilled in the art, for instance by comparing treated or placebo-treated age-matched subjects.

15

Example 19: Modified Method for Preparing Amyloid β Oligomers

This Example describes an alternative method for making ADDLs that can be employed instead of, for instance, the methods described in Examples 1 and 4.

Amyloid β monomer stock solution is made by dissolving the monomer
20 in hexafluoroisopropanol (HFIP), which is subsequently removed by speed vacuum evaporation. The solid peptide is redissolved in dry DMSO at 5 mM to form a DMSO stock solution, and the ADDLs are prepared by diluting 1 μ l of the DMSO stock solution into 49 μ l of F12 media (serum-free, phenol-red free). The mixture is vortexed and then incubated at 4°C for 24 hours.

25

Example 20: Further Gel Studies of Amyloid β Oligomers

This Example describes further gel studies done on amyloid β oligomers.

For gel analysis following preparation of the amyloid β oligomers (i.e.,

oligomers prepared as described in the prior example), 1 μ l of the oligomer solution is added to 4 μ l of F12 and 5 μ l of tris-tricine loading buffer, and then loaded on a pre-made 16.5% tris-tricine gel (Biorad). Electrophoresis is carried out for 2.25 hours at 100 V. Following electrophoresis, the gel is stained using the Silver Xpress kit (Novex). Alternately, instead of staining the gel, the amyloid β species are transferred from the gel to Hybond-ECL (Amersham) in SDS-containing transfer buffer for 1 hour at 100 V at 4°C. The blot is blocked in TBS-T1 containing 5% milk for 1 hour at room temperature. Following washing in TBS-T1, the blot is incubated with primary antibody (26D6, 1:2000,) for 1.5 hours at room temperature. The 26D6 antibody recognizes the amino terminal region of amyloid β . Following further washing, the blot is incubated with secondary antibody (anti-mouse HRP, 1:3500) for 1.5 hours at room temperature. Following more washing, the blot is incubated in West Pico Supersignal reagents (500 μ l of each, supplied by Pierce) and 3 mls of ddH₂O for 5 minutes. Finally, the blot is exposed to film and developed.

Results of such further gel studies are depicted in Figure 16, which shows a computer-generated image of a densitometer-scanned 16.5% tris-tricine SDS-polyacrylamide gel (Biorad). The figure confirms a range of oligomeric, soluble ADDLs (labeled "ADDLs"), dimer (labeled "Dimer"), and monomer (labeled "Monomer"). This gel system thus enables visualization of distinct ADDLs comprising from at least 3 monomers (trimer) up to about 24 monomers.

What is not depicted in Figure 16, but which becomes apparent upon comparing gels/Westerns obtained before and after aggregation is the fact that the tetramer band increases upon aggregation, whereas the pentamer through the 24-mer oligomer species appear only after aggregation. The differences between the silver stained and the immunodetected amounts of the oligomers (especially dimer and tetramer) suggest that the oligomers may represent different conformations obtained upon aggregation.

Example 21: Further AFM Studies of Amyloid β Oligomers

This Example describes further AFM studies done on amyloid β oligomers.

AFM was done as described in Example 3 except that fractionation on a Superdex 75 column was not performed, and the field was specifically selected such that larger size globules in the field were measured. The analysis is the same from a technical standpoint as that done in Example 3, but in this instance the field that was specifically selected for and examined allows visualization of oligomers that have larger sizes than those that were measured by the section analysis. AFM was carried out using a NanoScope® III MultiMode AFM (MMAFM) workstation using
10 TappingMode® (Digital Instruments, Santa Barbara, CA).

The results of these studies are shown in Figure 17, which is a computer-generated image of an AFM analysis of ADDLs showing various sized structures of different amyloid β oligomers. The adhered structures range in size from 1 to 10.5 nm in z height. Based on this characterization, the structures comprise from 3 to 24
15 monomeric subunits, consistent with the bands shown on Tris-tricine SDS-PAGE. In separate experiments (not shown) species as high as about 11 nm have been observed.

All of the references cited herein, including patents, patent applications, publications, and the like, are hereby incorporated in their entireties by reference.

20 While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments can be used, and that it is intended that the invention can be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the
25 invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. An isolated soluble non-fibrillar amyloid β oligomeric structure
5 comprising from about 3 to about 24 amyloid β proteins that does not contain an
exogenous added crosslinking agent and which exhibits neurotoxic activity.
2. , An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure comprises trimer, tetramer, pentamer, hexamer, heptamer,
10 octamer, 12-mer, 16-mer, 20-mer, or 24-mer aggregates of amyloid β proteins.
3. An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure has a molecular weight of from about 36 kD to about 108 kD as
determined by non-denaturing gel electrophoresis.
15
4. An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure has a molecular weight of from about 26 kD to about 28 kD as
determined by non-denaturing gel electrophoresis.
- 20 5. An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure has a molecular weight of from about 22 kD to about 24 kD as
determined by electrophoresis on a 15% SDS-polyacrylamide gel.
6. An isolated oligomeric structure according to claim 1 wherein said
25 oligomeric structure has a molecular weight of from about 18 kD to about 19 kD as
determined by electrophoresis on a 15% SDS-polyacrylamide gel.
7. An isolated oligomeric structure according claim 1 wherein said

oligomeric structure comprises globules of dimensions of from about 4.7 nm to about 11.0 nm as measured by atomic force microscopy.

8. An isolated oligomeric structure according to claim 1 wherein said
5 oligomeric structure comprises globules of dimensions of from about 4.7 nm to about 6.2 nm as measured by atomic force microscopy.

9. An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure comprises globules of dimensions of from about 4.9 nm to about
10 5.4 nm as measured by atomic force microscopy.

10. An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure comprises globules of dimensions of from about 5.7 nm to about 6.2 nm as measured by atomic force microscopy.
15

11. An isolated oligomeric structure according to claim 1 wherein said
oligomeric structure comprises globules of dimensions of from about 6.5 nm to about 11.0 nm as measured by atomic force microscopy.

20 12. An isolated oligomeric structure according to claim 1 wherein from about 40% to about 75% of said oligomeric structure comprises globules of dimensions of from about 4.9 nm to about 5.4 nm, and dimensions of from about 5.7 nm to about 6.2 nm, as measured by atomic force microscopy.

25 13. An isolated oligomeric structure according to claim 1 wherein said oligomeric structure has a molecular weight of from about 13 kD to about 116 kD as determined by electrophoresis on a 16.5% tris-tricine SDS-polyacrylamide gel.

14. An isolated oligomeric structure according to claim 1 wherein said oligomeric structure has, as determined by electrophoresis on a 16.5% tris-tricine SDS-polyacrylamide gel, a molecular weight selected from the group consisting of from about 13 kD to about 14 kD, from about 17 kD to about 19 kD, from about 22
5 kD to about 23 kD, from about 26 kD to about 28 kD, from about 32 kD to about 33 kD, and from about 36 kD to about 38 kD.

15. A method for assaying the effects of an oligomeric structure according to claim 1 comprising:

- 10 (a) administering said oligomeric structure to the hippocampus of an animal;
- (b) applying an electrical stimulus; and
- (c) measuring the cell body spike amplitude over time to determine the long-term potentiation response.

15

16. The method of claim 15, wherein the long-term potentiation response of said animal is compared to the long-term potentiation response of another animal treated in the same fashion except having saline administered instead of oligomeric structure prior to application of said electrical stimulus.

20

17. A method for protecting an animal against decreases in learning or memory due to the effects of a soluble non-fibrillar amyloid β oligomeric structure according to claim 1, said method comprising administering a compound that blocks the formation or activity of said oligomeric structure.

25

18. A method for reversing in an animal decreases in learning or memory due to the effects of a soluble non-fibrillar amyloid β oligomeric structure according to claim 1, said method comprising administering a compound that blocks the

formation or activity of said oligomeric structure.

19. A method for protecting a nerve cell against decreases in long-term
potentiation due to the effects of a soluble non-fibrillar amyloid β oligomeric structure
5 according to claim 1, said method comprising contacting said cell with a compound
that blocks the formation or activity of said oligomeric structure.

20. A method for reversing in a nerve cell decreases in long-term
potentiation due to the effects of a soluble non-fibrillar amyloid β oligomeric structure
10 according to claim 1, said method comprising contacting said cell with a compound
that blocks the formation or activity of said oligomeric structure.

21. A method for protecting a nerve cell against aberrant neuronal
signaling due to the effects of a soluble non-fibrillar amyloid β oligomeric structure
15 according to claim 1, said method comprising contacting said cell with a compound
that blocks the formation or activity of said oligomeric structure.

22. A method for detecting in a test material the oligomeric structure
according to claim 1 comprising:

- 20 (a) contacting said test material with 6E10 antibody; and
- - - - - (b) - detecting binding to said oligomeric structure of said antibody.

23. A method for detecting in a test material the oligomeric structure
according to claim 1 comprising:

- 25 (a) contacting said test material with serum-starved neuroblastoma cells;
and
(b) measuring morphological changes in said cells by comparing the
morphology of said cells against neuroblastoma cells that have not been contacted

with said test material.

24. A method for detecting in a test material the oligomeric structure according to claim 1 comprising:

- 5 (a) contacting said test material with brain slice cultures; and
(b) measuring brain cell death as compared against brain slice cultures that have not been contacted with said test material.

25. A method for detecting in a test material the oligomeric structure according to claim 1 comprising:

- 10 (a) contacting said test material with neuroblastoma cells; and
(b) measuring increases in Fyn kinase activity by comparing Fyn kinase activity in said cells against Fyn kinase activity in neuroblastoma cells that have not been contacted with said test material.

15 26. A method for detecting in a test material the oligomeric structure according to claim 1 comprising:

- (a) contacting said test material with cultures of primary astrocytes; and
(b) determining activation of said astrocytes as compared to cultures of
20 primary astrocytes that have not been contacted with said test material.

27. A method for detecting in a test material the oligomeric structure according to claim 1 comprising:

- (a) contacting said test material with cultures of primary astrocytes; and
25 (b) measuring in said astrocytes increases in the mRNA for proteins selected from the group consisting of interleukin-1, inducible nitric oxide synthase, Apo E, Apo J, and α 1-antichymotrypsin by comparing said mRNA levels in said astrocytes against the corresponding mRNA levels in cultures of primary astrocytes

that have not been contacted with said test material.

28. A method for identifying compounds that modulate the effects of an oligomeric structure according to claim 1 comprising:

- 5 (a) administering either saline or a test compound to the hippocampus of an animal;
- (b) applying an electrical stimulus;
- (c) measuring the cell body spike amplitude over time to determine the long-term potentiation response; and
- 10 (d) comparing the long-term potentiation response of animals having saline administered to the long-term potentiation response of animals having test compound administered

with the proviso that administration of said oligomeric structure is not done for therapy.

15

29. The method of claim 28 which further comprises administering oligomeric structure to said hippocampus either before, along with, or after administering said saline or test compound.

20 30. A method for identifying compounds that block the neurotoxicity of the oligomeric structure according to claim 1 comprising:

- (a) contacting separate cultures of neuronal cells with said oligomeric structure either in the presence or absence of contacting with said test compound;
- (b) measuring the proportion of viable cells in each culture; and
- 25 (c) comparing the proportion of viable cells in each culture, with compounds that block the neurotoxicity of said oligomeric structure being identified as resulting in an increased proportion of viable cells in said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of

said test compound.

31. A method for identifying compounds that block binding to a cell surface protein of the oligomeric structure according to claim 1 comprising:

- 5 (a) contacting separate cultures of neuronal cells with said oligomeric structure either in the presence or absence of contacting with said test compound;
- (b) adding a reagent that binds to said oligomeric structure, said reagent being fluorescent;
- (c) analyzing said separate cell cultures by fluorescence-activated cell
- 10 sorting; and
- (d) comparing the fluorescence of the cultures, with compounds that block binding to a cell surface protein of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound.

15

32. A method for identifying compounds that block binding to a cell surface protein of the oligomeric structure according to claim 1 comprising:

- (a) forming said oligomeric structure from amyloid β protein such that it becomes a labeled oligomeric structure comprising a binding moiety capable of
- 20 binding a fluorescent reagent;
- (b) contacting separate cultures of neuronal cells with said labeled oligomeric structure either in the presence or absence of contacting with said test compound;
- (c) adding a fluorescent reagent that binds to said oligomeric structure;
- 25 (d) analyzing said separate cell cultures by fluorescence-activated cell sorting; and
- (e) comparing the fluorescence of the cultures, with compounds that block binding to a cell surface protein of the oligomeric structure being identified as

resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound.

33. A method for identifying compounds that block formation or binding
5 to a cell surface protein of the oligomeric structure according to claim 1 comprising:
- (a) preparing separate samples of amyloid β protein that either have or have not been mixed with said test compound;
 - (b) forming said oligomeric structure in said separate samples;
 - (c) contacting separate cultures of neuronal cells with said separate
10 samples;
 - (d) adding a reagent that binds to said oligomeric structure, said reagent being fluorescent;
 - (e) analyzing said separate cell cultures by fluorescence-activated cell sorting; and
 - 15 (f) comparing the fluorescence of the cultures, with compounds that block formation or binding to a cell surface protein of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound.

20

- 34. A method for identifying compounds that block formation or binding to a cell surface protein of the oligomeric structure according to claim 1 comprising:
- (a) preparing separate samples of amyloid β protein that either have or have not been mixed with said test compound;
 - 25 (b) forming said oligomeric structure in said separate samples such that it becomes a labeled oligomeric structure comprising a binding moiety capable of binding a fluorescent reagent in each of said separate samples;
 - (c) contacting separate cultures of neuronal cells with said separate

samples;

(d) adding a fluorescent reagent that binds to said oligomeric structure;

(e) analyzing said separate cell cultures by fluorescence-activated cell sorting; and

5 (f) comparing the fluorescence of the cultures, with compounds that block formation or binding to a cell surface protein of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound.

10

35. The method of claim 33, wherein the fluorescence of said cultures further is compared with the fluorescence of cultures that have been treated in the same fashion except that instead of adding or not adding test compound prior to formation of the oligomeric structure, said test compound either is or is not added
15 after formation of the oligomeric structure,

with compounds that block formation of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound, only when said compound is added prior to oligomeric structure, and

20 compounds that block binding to a cell surface protein of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound, when said compound is added either prior to or after oligomeric structure.

25

36. The method of claim 33, wherein the fluorescence of said cultures further is compared with the fluorescence of cultures that have been treated in the same fashion except that instead of adding or not adding test compound prior to

formation of the oligomeric structure; said test compound either is or is not added after formation of the oligomeric structure,

with compounds that block formation of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the
5 corresponding culture contacted with said oligomeric structure in the absence of said test compound, only when said compound is added prior to oligomeric structure, and

compounds that block binding to a cell surface protein of the oligomeric structure being identified as resulting in a reduced fluorescence of said culture as compared to the corresponding culture contacted with said oligomeric structure in the
10 absence of said test compound, when said compound is added either prior to or after oligomeric structure.

37. A method of detecting binding to a cell surface protein of the oligomeric structure according to claim 1 comprising:

- 15 (a) forming said oligomeric structure from amyloid β protein;
(b) contacting a culture of neuronal cells with said oligomeric structure;
(c) adding an antibody that binds said oligomeric structure, said antibody including a conjugating moiety;
(e) washing away unbound antibody;
20 (f) linking an enzyme to said antibody bound to said oligomeric structure by means of said conjugating moiety;
(g) adding a colorless substrate that is cleaved by said enzyme to yield a color change; and
(h) determining said color change as a measure of binding to a cell surface
25 protein of said oligomeric structure.

38. A method for identifying compounds that block binding to a cell surface protein of the oligomeric structure according to claim 1 comprising:

- (a) preparing separate samples of amyloid β protein that either have or have not been mixed with said test compound;
- (b) forming said oligomeric structure in said separate samples;
- (c) contacting separate cultures of neuronal cells with said separate
5 samples;
- (d) adding an antibody that binds said oligomeric structure, said antibody including a conjugating moiety;
- (e) washing away unbound antibody;
- (f) linking an enzyme to said antibody bound to said oligomeric structure
10 by means of said conjugating moiety;
- (g) adding a colorless substrate that is cleaved by said enzyme to yield a color change; and
- (h) comparing the color change produced by each of said separate samples, with compounds that block formation or binding to a cell surface protein of the
15 oligomeric structure being identified as resulting in a reduced color change produced by said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound.

39. The method of claim 38, wherein the color change produced by said
20 cultures further is compared with the color change produced by cultures that have been treated in the same fashion except that instead of adding or not adding test compound prior to formation of the oligomeric structure, said test compound either is or is not added after formation of the oligomeric structure,
- with compounds that block formation of the oligomeric structure being
25 identified as resulting in a reduced color change produced by said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound, only when said compound is added prior to oligomeric structure, and

compounds that block receptor binding of the oligomeric structure being identified as resulting in a reduced color change produced by said culture as compared to the corresponding culture contacted with said oligomeric structure in the absence of said test compound, when said compound is added either prior to or after oligomeric

5 structure.

40. A method for identifying compounds that block formation of the oligomeric structure according to claim 1 comprising:

- 10 (a) preparing separate samples of amyloid β protein that either have or have not been mixed with said test compound;
- (b) forming said oligomeric structure in said separate samples;
- (c) assessing whether any protein assemblies have formed in the separate samples using a method selected from the group consisting of electrophoresis, immunorecognition, and atomic force microscopy; and
- 15 (d) comparing the formation of said protein assemblies in said separate samples, which compounds that block formation of said oligomeric structure being identified as resulting in decreased formation of said oligomeric structure in said sample as compared with a sample in which said oligomeric structure is formed in the absence of said test compound.

20

41. A method of preparing an isolated soluble, globular, non-fibrillar amyloid β oligomeric structure according to claim 1, wherein said method comprises:

- (a) obtaining a solution of monomeric amyloid β protein, said amyloid β protein being capable of forming said oligomeric structure;
- 25 (b) diluting said protein solution into an appropriate media to a final concentration of from about 5 nM to about 500 μ M;
- (c) incubating the media resulting from step (b) at about 4°C for from about 2 hours to about 48 hours;

- (c) centrifuging said solution at about 14,000 g at about 4°C; and
- (d) recovering the supernatant resulting from said centrifugation as containing said amyloid β oligomeric structure.

5 42. The method of claim 41, wherein said method comprises incubating the media resulting from step (b) at about 4°C in the presence of clusterin.

 43. A method for preparing a soluble non-fibrillar amyloid β oligomeric structure according to claim 1, wherein said method comprises:

- 10 (a) obtaining a solution of monomeric amyloid β protein, said amyloid β protein being capable of forming said oligomeric structure;
- (b) dissolving said amyloid β monomer in hexafluoroisopropanol;
- (c) removing hexafluoroisopropanol by speed vacuum evaporation to obtain solid peptide;
- 15 (d) dissolving said solid peptide in DMSO to form a DMSO stock solution;
- (e) diluting said stock solution into an appropriate media;
- (f) vortexing; and
- (g) incubating at about 4°C for about 24 hours.

20

-----44. A method for detecting in a test material the oligomeric structure according to claim 1 comprising:

- (a) contacting said test material with a nerve cell; and
- determining whether said cell exhibits ADDL-induced aberrant neuronal signaling.

25



Figure 1

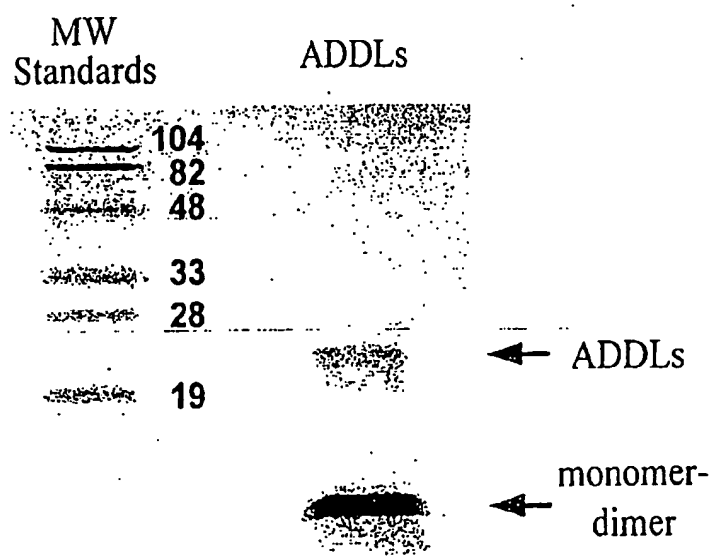


Figure 2

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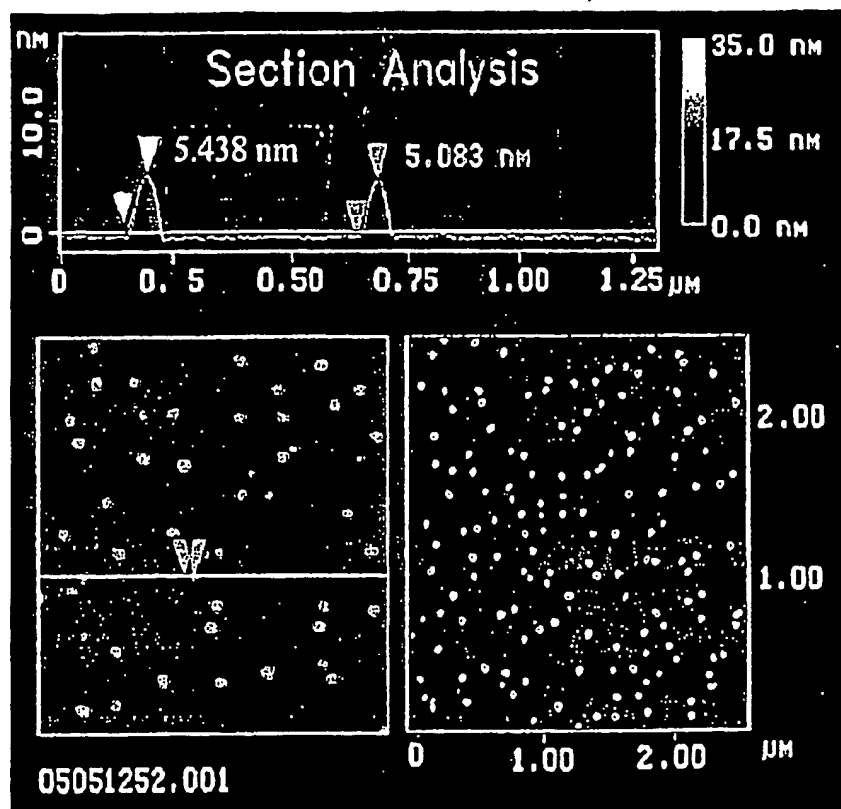


Figure 3

3/10

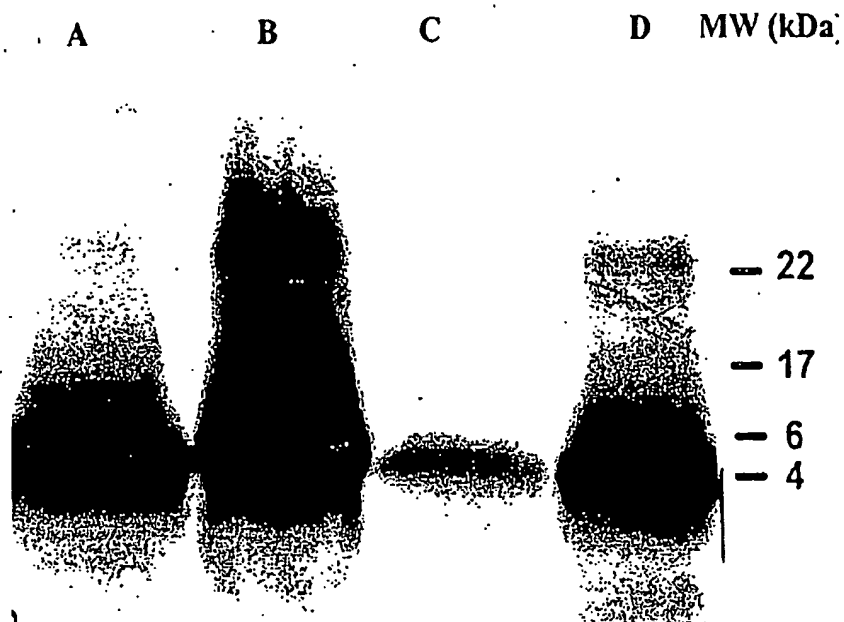


Figure 4

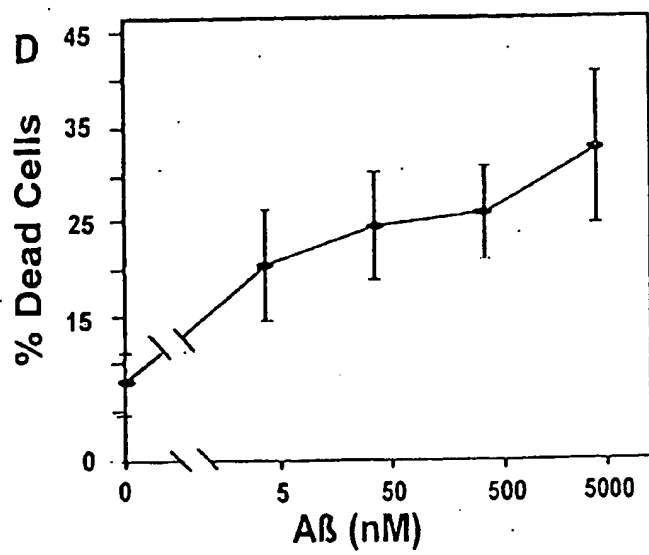


Figure 5

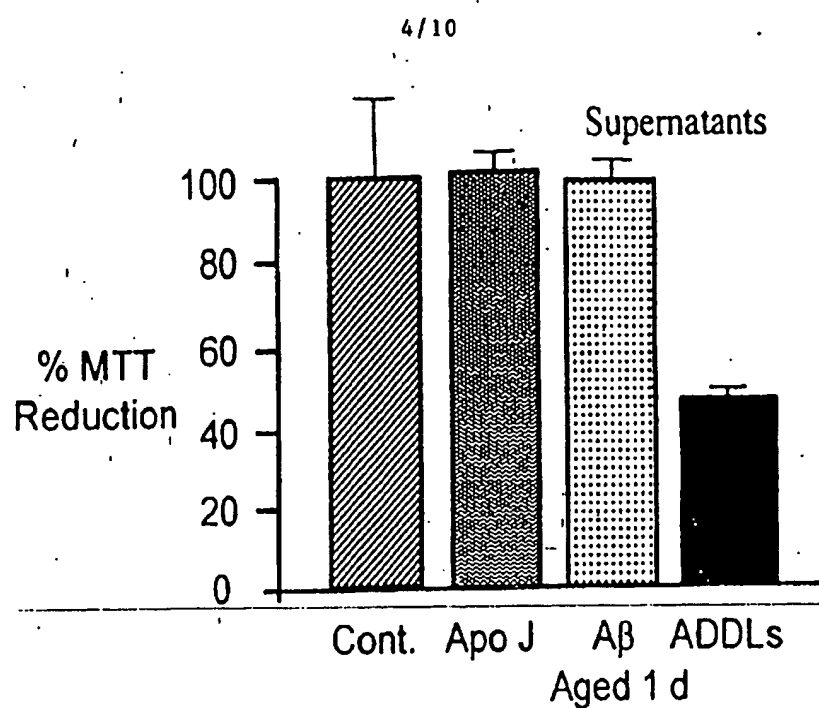


Figure 6

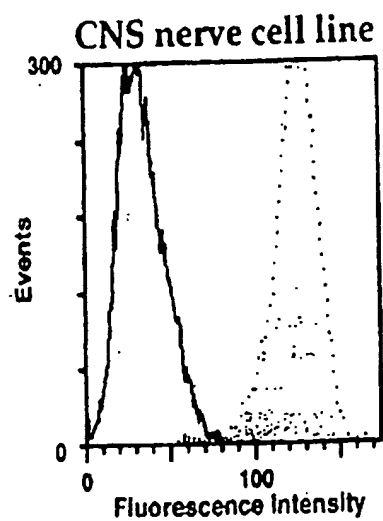


Figure 7

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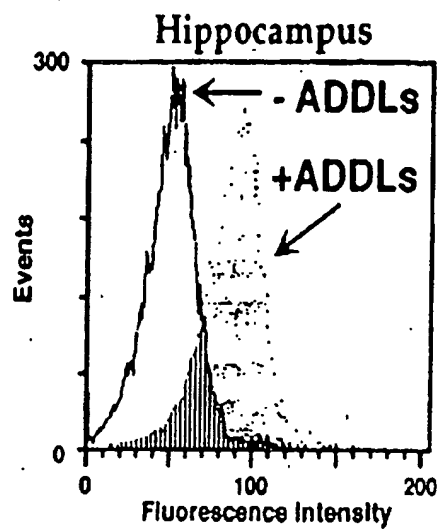


Figure 8

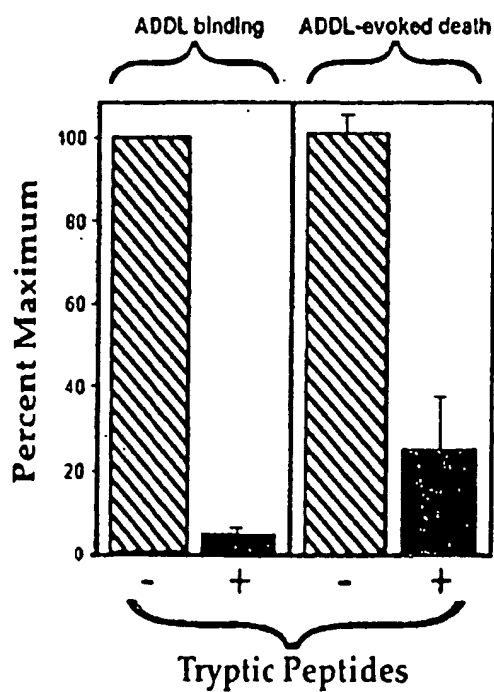


Figure 9

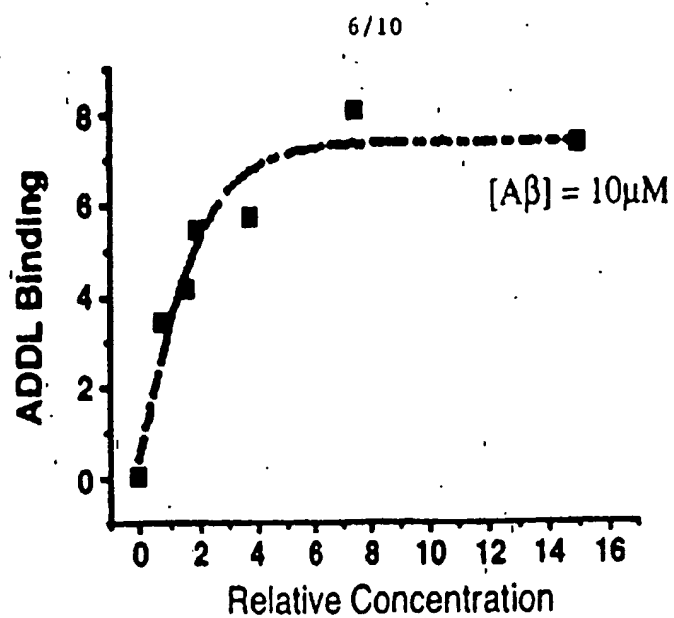


Figure 10

ADDL Binding ELISA

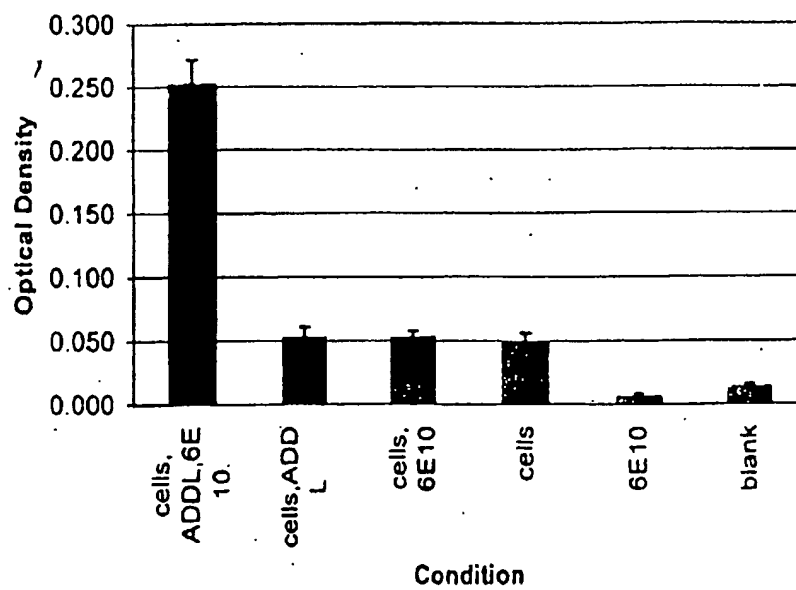


Figure 11

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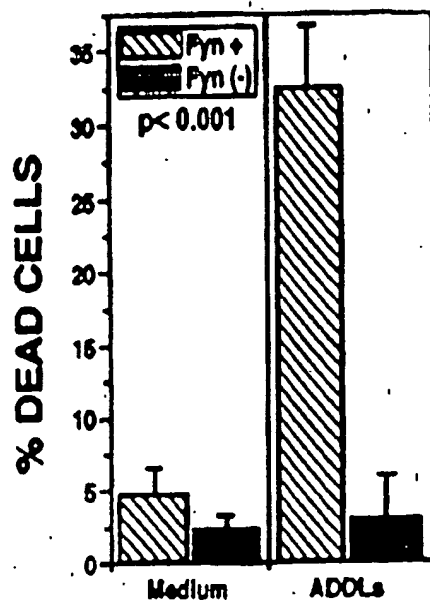


Figure 12

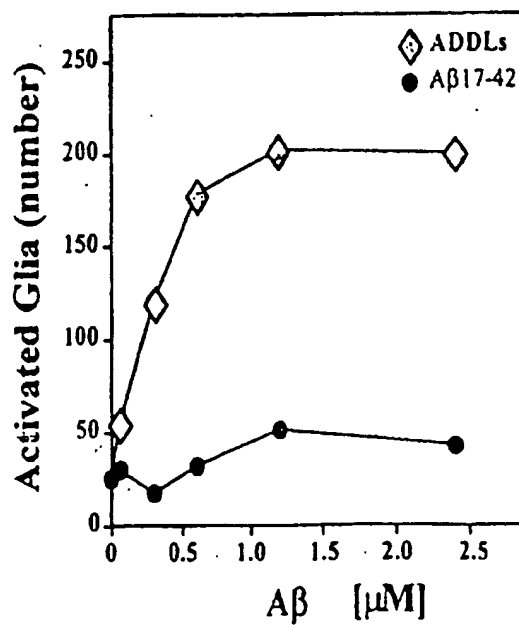


Figure 13

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Cell Body Spike Amplitude

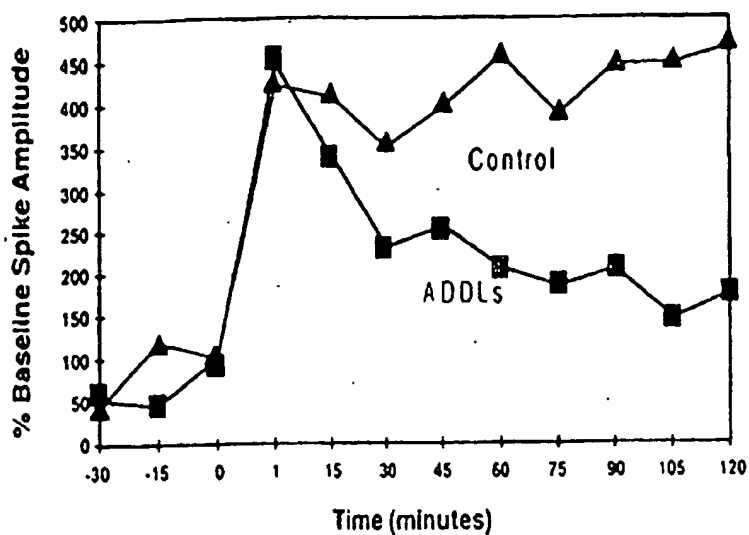


Figure 14

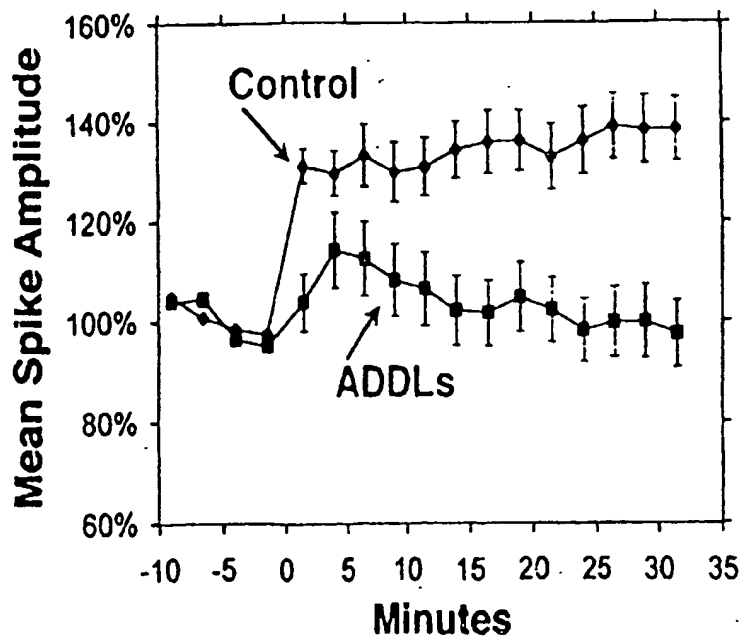


Figure 15

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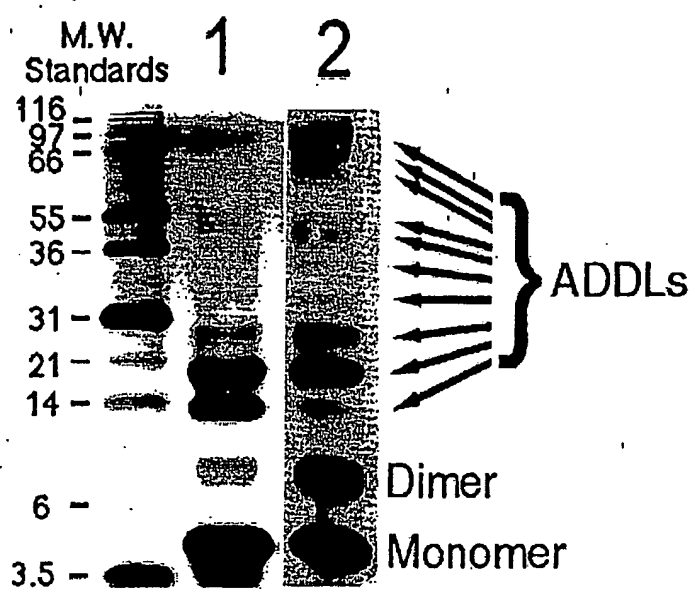


Figure 16

10/10

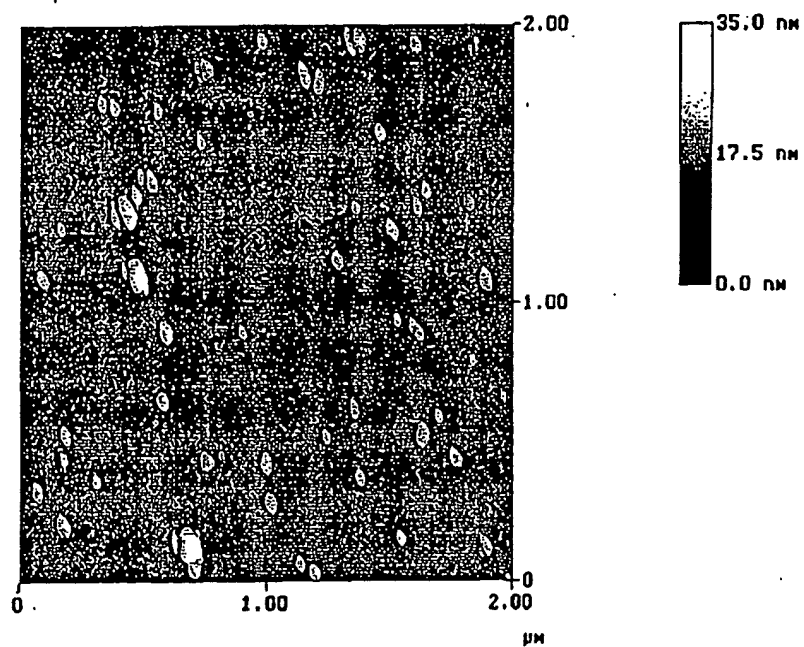


Figure 17

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Grant A. Krafft, William L. Klein, Brett A. Chromy,
Mary P. Lambert, Caleb E. Finch, Todd Morgan, Pat Wals,
Irina Rozovsky, Ann Barlow

(ii) TITLE OF INVENTION: Amyloid β Protein (Globular Assembly
and Uses Thereof)

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (US)

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(A) APPLICATION NUMBER: unassigned
(B) FILING DATE: 04-AUG-1999
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(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/796,089
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCACCTTCTT TCCCTTCATC

20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGCTGATGTA CCAGTTGGGG

20

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CAGTCCTTGA CCTGCGACC

19

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCTCACATC ACATCCTTG

19



GP/1632

| TRANSMITTAL LETTER | | | Case No. 8792/293 |
|--|--------------------------------|----------|------------------------|
| Serial No. 10/226,435 | Filing Date August 22, 2002 | Examiner | Group Art Unit 1632 |
| Inventor(s) David M. Holtzman, et al. | | | |
| Title of Invention Humanized Antibodies That Sequester Amyloid Beta Peptide | | | |

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is Information Disclosure Statement (2), Form PTO-1449, Copies of Cited References A1-A85, Postcard Receipt,

- ☐ Small entity status of this application under 37 CFR § 1.27 has been established by verified statement previously submitted.
- ☐ A verified statement to establish small entity status under 37 CFR §§ 1.9 and 1.27 is enclosed.
- ☐ Petition for a month extension of time.
- ☒ No additional fee is required.
- ☐ The fee has been calculated as shown below:

| | | | | Small Entity | | or | Other Than Small Entity | |
|---|----------------------------------|-------|---------------------------------|---------------|-----------------|-----------|-------------------------|-----------|
| | Claims Remaining After Amendment | | Highest No. Previously Paid For | Present Extra | Rate | Add'l Fee | Rate | Add'l Fee |
| Total | | Minus | | | x \$9 = | | x \$18 = | |
| Indep. | | Minus | | | x \$42 = | | x \$84 = | |
| First Presentation of Multiple Dep. Claim | | | | | + \$140 = | | + \$280 = | |
| | | | | | Total add'l fee | \$ | Total add'l fee | |

- ☐ Please charge Deposit Account No. 23-1925 (BRINKS HOFER GILSON & LIONE) in the amount of \$ _____. A duplicate copy of this sheet is enclosed.
- ☐ A check in the amount of \$ _____ to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR § 1.16 and any patent application processing fees under 37 CFR § 1.17 associated with this communication or credit any overpayment to Deposit Account No. 23-1925. A duplicate copy of this sheet is enclosed.
- ☒ I hereby petition under 37 CFR § 1.136(a) for any extension of time required to ensure that this paper is timely filed. Please charge any associated fees which have not otherwise been paid to Deposit Account No. 23-1925. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

John Murray
John Murray
Registration No. 44,251
Attorney for Applicant

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail, with sufficient postage, in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on December 17, 2002.

Date: December 17, 2002

Signature: John Murray

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CERTIFICATE OF MAILING

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John Murray

Name of Applicant, Assignee or
Registered Representative

John Murray
Signature

December 17, 2002

Date of Signature

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Our Case No.: 8792/293

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

David M. Holtzman et al.

Serial No.: 10/226,435

Filing Date: August 22, 2002

For: HUMANIZED ANTIBODIES THAT
SEQUESTER A β PEPTIDE

Examiner:

Group Art Unit No.: 1632

INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In compliance with the duty of disclosure under 37 C.F.R. § 1.56, it is respectfully requested that this Information Disclosure Statement be entered and the documents listed below and on the attached Form PTO-1449 be considered by the Examiner and



made of record. Copies of the listed documents required by 37 C.F.R. § 1.98(a)(2) are enclosed for the convenience of the Examiner.

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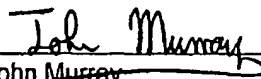
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In accordance with 37 C.F.R. § 1.97(g),(h), this Information Disclosure Statement is not to be construed as a representation that a search has been made and is not to be construed to be an admission that the information cited is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

Since it is believed that this Information Disclosure Statement is being filed prior to the mailing of the first Official Action reflecting an examination on the merits and hence is believed to be timely filed in accordance with 37 C.F.R. § 1.97(b). No fees are believed to be due in connection with filing of this Information Disclosure Statement, however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be deemed necessary for any reason relating to these materials, the Commissioner is hereby authorized to deduct said fees from Brinks Hofer Gilson & Lione Deposit Account No. 23-1925. A duplicate copy of this document is enclosed.

Applicants respectfully request that the listed documents be made of record in the present case.

Respectfully submitted,


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54 Pharmaceutical screens and antibodies.

57 This invention describes a series of assays useful in evaluating the efficacy of agents which inhibit the neurotoxic effects of β -amyloid peptide. These assays employ β -amyloid peptide which is in a predominantly β -sheet conformation. This invention also encompasses antibodies having a specificity for β -amyloid peptide which is predominantly in a β -sheet conformation as well as pharmaceutical formulations containing these antibodies. These antibodies show poor reactivity with β -amyloid peptide which has a great deal of random coil or α -helix secondary structure.

EP 0 613 007 A2

Alzheimer's disease is a degenerative disorder of the human brain. Clinically, it appears as a progressive dementia. Its histopathology is characterized by degeneration of neurons, gliosis, and the abnormal deposition of proteins in the brain. Proteinaceous deposits (called "amyloid") appear as neurofibrillary tangles, amyloid plaque cores, and amyloid of the congophilic angiopathy. [For reviews, see, Alzheimer's Disease, (B. Reisberg, ed., The Free Press 1983).]

While there is no general agreement as to the chemical nature of neurofibrillary tangles, the major constituent of both the amyloid plaque cores and the amyloid of the congophilic angiopathy has been shown to be a 4500 Dalton protein originally termed β -protein or amyloid A4. Throughout this document this protein is referred to as β -amyloid peptide or protein.

β -amyloid peptide is proteolytically derived from a transmembrane protein, the amyloid precursor protein. Different splice forms of the amyloid precursor protein are encoded by a widely expressed gene. see, e.g., K. Beyreuther and B. Müller-Hill, Annual Reviews in Biochemistry, 58:287-307 (1989). β -amyloid peptide consists, in its longest forms, of 42 or 43 amino acid residues. J. Kang, et al., Nature (London), 325:733-736 (1987). These peptides, however, vary as to their amino-termini. C. Hilbich, et al., Journal of Molecular Biology, 218:149-163 (1991).

Because senile plaques are invariably surrounded by dystrophic neurites, it was proposed early that β -amyloid peptide is involved in the loss of neuronal cells that occurs in Alzheimer's disease. B. Yankner and co-workers were the first to demonstrate that synthetic β -amyloid peptide could be neurotoxic in vitro and in vivo. B.A. Yankner, et al., Science, 245:417 (1989); See, also, N.W. Kowall, et al., Proceedings of the National Academy of Sciences, U.S.A., 88:7247 (1991). Other research groups, however, were unable to consistently demonstrate direct toxicity with β -amyloid peptide. See, e.g., Neurobiology of Aging, 13:535 (K. Kosik and P. Coleman, eds. 1992). Even groups receiving β -amyloid peptide from a common source demonstrate conflicting results. D. Price, et al., Neurobiology of Aging, 13:623-625 (1991) (and the references cited therein).

Because of the debilitating effects of Alzheimer's disease there continues to exist a need for effective treatments. This invention provides assay systems which are useful to evaluate the efficacy of potential agents to treat this disease.

This invention describes a series of assays useful in evaluating the efficacy of agents which inhibit the neurotoxic effects of β -amyloid peptide. These assays employ β -amyloid peptide which is in a predominantly β -sheet conformation.

In another embodiment, this invention describes antibodies having a specificity for β -amyloid peptide

which is predominantly in a β -sheet conformation. These antibodies show poor reactivity with β -amyloid peptide which has a high degree of random coil or α -helix secondary structure.

This invention also encompasses pharmaceutical formulations comprising an antibody having a specificity for β -amyloid peptide which is predominantly in a β -sheet conformation in combination with a parenterally-administrable medium.

The terms and abbreviations used in the instant examples have their normal meanings unless otherwise designated. For example "°C" refers to degrees Celsius; "N" refers to normal or normality; "mmole" refers to millimole or millimoles; "g" refers to gram or grams; "ml" means milliliter or milliliters; "M" refers to molar or molarity; and "CD" refers to circular dichroism spectrometry.

This invention depicts assays employing β -amyloid peptide in which the secondary structure of the peptide is predominantly β -sheet. The amount of β -sheet structure present in the β -amyloid peptides employed in this specification differs.

A preferred embodiment of this invention employs β -amyloid peptide which has adopted at least 65% of its potential β -sheet conformation. The most preferred embodiment of this invention employs β -amyloid peptide which has adopted at least 80% of its potential β -sheet conformation. The amount of β -sheet conformation present in a peptide lot can be readily determined by examining the circular dichroism spectrum of the peptide lot.

While this peptide is referred to as β -amyloid peptide throughout this document, in the body of literature concerning this field this peptide is alternatively referred to as β -amyloid protein, amyloid β peptide, amyloid β A4, β protein, amyloid A4, β -peptide, and other such names.

β -amyloid peptide naturally occurs as a series of peptides which are 39 to 43 amino acids long, with the shorter, more soluble forms being present in cerebrovascular deposits and the longer forms being found primarily in senile plaques. F. Prelli, et al., Journal of Neurochemistry, 51:648-651 (1988).

Even though the full length peptide has sufficient solubility in water for the following experiments, for the purposes of convenience, a more water-soluble form of the peptide is often desired. For that reason, the following examples were performed using peptides containing just the first 40 amino acids of the β -amyloid peptide (β 1-40).

It is understood by those in the art that other fragments of β -amyloid peptide, comprising amino-truncated, carboxy-truncated, or internal deletions, or any combination of these, may be employed in this invention so long as that peptide fragment demonstrates the requisite neurotoxicity.

The most frequently employed method to ensure that β -amyloid peptide adopts a β -sheet secondary

structure involves "aging" of the peptide. This process comprises incubating the peptide in water or tissue culture medium for 1-10 days. The amount of time necessary for the peptide to assume a β -sheet secondary structure depends upon the constitution and pH of the buffer in which it is stored, the temperature at which the incubation occurs, the concentration of peptide in the solution and the length of the peptide employed.

Peptide Synthesis and Purification

The peptides employed in the instant invention can be prepared using any one of many different protocols or can be purchased from commercial sources. One synthesis protocol employs stepwise solid phase peptide synthesis using a mechanical peptide synthesizer. I. Clark-Lewis, et al., *Science*, 231:134-139 (1986). This procedure uses commercially available *N*-*t*-butoxycarbonyl-protected amino acids and phenylacetamidomethyl or *p*-methylbenzhydrylamine supporting resins. The side chain blocking groups which are commonly used include: *O*-benzyl groups for aspartate, serine, glutamic acid, and threonine; 2-chlorocarbobenzyloxy moieties for blocking lysine; bromocarbobenzyloxy groups for tyrosine; tosyl groups for blocking arginine and histidine; and acetamidomethyl for cysteine. No side-chain protection is necessary for asparagine, glutamine, or methionine.

Peptides are deprotected and cleaved from the supporting resin by reaction with liquid hydrogen fluoride (in the presence of anisole) for about 1 hour at 0°C, precipitated in diethyl ether and extracted from the resin with formic acid (about 70%, v/v). To purify and desalt the peptides, crude extracts are chromatographed on size-exclusion columns equilibrated with 70% formic acid.

The peptide content of the eluate is monitored by measurement of its absorption at 280 nm. Fractions within the appropriate molecular weight range of the peptide monomer are pooled and lyophilized. A second purification is done by size-exclusion chromatography in 1 M acetic acid.

An alternative method of purifying the peptides is by the use of reversed-phase high performance liquid chromatography (RP-HPLC). The peptides are loaded onto a nonpolar stationary phase using a solvent such as 0.1% trifluoroacetic acid in water. The peptides are then eluted from the column using a gradient of increasing polarity, such as increasing concentrations of acetonitrile.

The identity and purity of the peptide is then checked using Edman degradation. The Edman degradation may be performed on whole peptides, cyanogen bromide-cleaved peptides, or protease-generated peptides. The Edman degradation may be supplemented with amino acid analysis. Alternatively the identity and purity of the synthesized peptides can be

determined using mass spectrometric techniques such as electrospray, laser desorption or fast atom bombardment.

Circular Dichroism Spectroscopy

The circular dichroism spectra can be determined using commonly known procedures. In one such procedure, solutions of peptides are mixed at room temperature for about 18 hours. If necessary, the solution is centrifuged at about 10,000 x g for about 10 minutes to produce a clear solution so that solubilized peptide can be analyzed. The CD measurements are made using a spectropolarimeter such as the AVIV 62DS. A 1 mm quartz cell is usually used for far-ultraviolet (190-240 nm) spectra. The instrument is calibrated frequently and base lines are determined. Multiple scans of each sample are obtained and the base lines deducted. Quantitative curve-fitting is done using standard reference sets. See, e.g., N. Greenfield and G. Fasman, *Biochemistry*, 8:4108-4116 (1969); M. Crisma, et al., *International Journal of Peptide and Protein Research*, 23:411-419 (1984).

Neurotoxicity Assay Measuring Calcium Levels

An aliquot of ED 18 cortical cells were seeded into polyethylenimine-coated tissue culture dishes for 3-5 days *in vitro* before treatment with a 25 μ M solution of β -amyloid peptide, either freshly dissolved (predominantly random coil conformation) or aged (7 days, predominantly β -sheet conformation). This neurotoxicity assay was conducted in chemically-defined HEPES-buffered DMEM supplemented with fetal calf serum.

After a two day incubation with the β -amyloid peptide, the elevation of cytosolic calcium (Ca^{+2}) concentrations after a glutamate pulse were determined using a fluorescent calcium dye. J. Wahl, et al., *Journal of Neurochemistry*, 53:1316 (1989). The elevation of intracellular Ca^{+2} levels compromises cell integrity.

Neurotoxicity Assay Measuring XTT

An aliquot of ED 18 cortical cells were seeded into polyethylenimine-coated tissue culture dishes for 3-5 days *in vitro* before treatment with a 25 μ M solution of β -amyloid peptide, either freshly dissolved (predominantly random coil conformation) or aged (7 days, predominantly β -sheet conformation). This assay was conducted in chemically-defined HEPES-buffered DMEM supplemented with fetal calf serum.

These cells were incubated for 3 to 5 days *in vitro* before treatment with a 25 μ M solution of β -amyloid peptide, either freshly dissolved (predominantly random coil conformation) or aged (7 days, predominantly β -sheet conformation). After two days of incubation, cell viability was assessed by measuring the

reduction of the tetrazolium salt XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt] as described by N. Roehm, et al., *Journal of Immunological Methods*, 142:257 (1992).

Neurotoxicity Assay Measuring LDH

Primary hippocampal cultures were prepared from E18 fetal Sprague-Dawley rat pups. The cells were plated at high density ($1.5 \times 10^5/\text{cm}^2$) in 24-well plates coated with 0.5 mg/ml of polyethylenimine. These cultures were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM glutamine, 5 g/liter glucose, 1 mM pyruvate, 20 mM potassium chloride, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin.

The cells were incubated for 10 days *in vitro* before treatment with a 25 μM solution of β -amyloid peptide, either freshly dissolved or aged. After four days of incubation, cell integrity was determined by measuring the level of the enzyme lactate dehydrogenase (LDH) using a standard colorimetric endpoint assay for pyruvate. P. May, et al., *Neurobiology of Aging*, 13:605, 606 (1992). Control standards containing normal and elevated levels of serum LDH were run with every assay.

The results of these neurotoxicity experiments demonstrates there is a direct correlation between the degree of β -sheet structure in the β -amyloid peptide and its neurotoxicity. There is minimal neurotoxicity associated with those samples of β -amyloid peptide that have a high degree of random coil in their secondary structure.

The use, therefore, of β -amyloid peptide which has adopted a predominantly β -sheet conformation allows the development of compounds which specifically inhibit the neurotoxicity. The neurotoxicity assays described, *supra*, can then be supplemented by the incubation of the β -amyloid peptide with potential inhibitors of neurotoxicity. The reduction in neurotoxicity can then be observed in an efficient manner.

Another embodiment of this invention encompasses conformationally-specific antibodies and antibody fragments which bind to β -amyloid peptides in a secondary structure-specific manner. Some of these antibodies bind only those β -amyloid peptides which are predominantly in a β -sheet conformation. A second set of these antibodies bind only those β -amyloid peptides which have adopted a random coil or α -helix conformation.

The term "antibody" as used in this specification describes antibodies, fragments of antibodies (such as, but not limited, to Fab, Fab', Fab₂', and Fv fragments), and chimeric, humanized, veneered, resurfaced, or CDR-grafted antibodies capable of binding antigens of a similar nature as the parent antibody molecule from which they are derived. The instant invention also encompasses single chain polypeptide

binding molecules.

The term "antibody" as used in this specification is not limited by the manner in which the antibodies are produced, whether such production is *in situ* or not. The term "antibody" as used in this specification encompasses those antibodies produced by recombinant DNA technology means including, but not limited, to expression in bacteria, yeast, insect cell lines, or mammalian cell lines.

The synthesis of antibodies, both monoclonal and polyclonal, in animals, especially mice, is well known in the art. See, e.g., C. Milstein, *Handbook of Experimental Immunology*, (Blackwell Scientific Pub., 1986); J. Coding, *Monoclonal Antibodies: Principles and Practice*, (Academic Press, 1983). Numerous species including rats, mice, rabbits, goats, and humans are useful as a source of immunized lymphocytes for the fusion protocols utilized to generate hybridomas. BALB/c mice are especially preferred as the source of the immune cells for production of the antibodies of this invention.

It is commonplace in the field of antibody production to screen for antibodies which show a high level of specificity for defined structure such as the β -amyloid peptide in a specific conformation, while showing markedly less specificity for the same peptide having a different secondary structure. The procedures for mass screenings of hybridomas are well known in the art. See, e.g., J. Starling, et al., *Cancer Immunology Immunotherapy*, 28:171 (1989).

The greatest deterrence to the administration to humans of antibodies produced in non-human sources is the risk of hyperimmunogenicity due to the presence of constant regions from the species in which these antibodies are produced. Genetically engineered antibodies which retain the epitope specificity of monoclonal antibodies are now known in the art and provide a less immunogenic molecule. Such genetically engineered antibodies are contemplated in the present invention.

Chimeric antibodies are described in U.S. Patent No. 4,816,567, which issued March 28, 1989 to S. Cabilly, et al. This reference discloses methods and vectors for the preparation of chimeric antibodies. The entire contents of U.S. Patent No. 4,816,567 are herein incorporated by reference. An alternative approach to production of genetically engineered antibodies is provided in U.S. Patent No. 4,816,397, which also issued March 28, 1989 to M. Boss, et al., the entire contents of which are herein incorporated by reference. The Boss patent teaches the simultaneous co-expression of the heavy and light chains of the antibody in the same host cell.

The approach of U.S. Patent 4,816,397 has been further refined as taught in European Patent Publication No. 0 239 400, which published September 30, 1987. The teachings of this European patent publication (Winter) are the preferred format for the genetic

engineering of the reactive monoclonal antibodies of this invention. The Winter technology involves the replacement of complementarity determining regions (CDRs) of a human antibody with the CDRs of a murine monoclonal antibody thereby converting the specificity of the human antibody to the specificity of the murine antibody which was the source of the CDR regions. This "CDR grafting" technology affords a molecule containing minimal murine sequence and thus is less immunogenic.

Single chain antibody technology is yet another variety of genetically engineered antibody which is now well known in the art. See, e.g. R.E. Bird, et al., *Science* 242:423-426 (1988); PCT Publication No. WO 88/01649, which was published 10 March 1988. The single chain antibody technology involves joining the binding regions of heavy and light chains with a polypeptide sequence to generate a single polypeptide having the binding specificity of the antibody from which it was derived.

The aforementioned genetic engineering approaches provide the skilled artisan with numerous means to generate molecules which retain the binding characteristics of the parental antibody while affording a less immunogenic format.

Some of the antibodies of the present invention demonstrate specificity for β -amyloid peptides which are predominantly β -sheet in conformation, as predominantly is defined *supra*. These antibodies show little binding specificity for β -amyloid peptides which have a great deal of random coil and/or α -helix in the secondary structure.

In another embodiment of this invention are antibodies which are specific for β -amyloid peptides which have adopted a random coil or α -helix conformation. These antibodies show little binding specificity for β -amyloid peptides which have a great deal of β -sheet conformation.

These antibodies are used in diagnostics, therapeutics or in diagnostic/therapeutic combinations. By "diagnostics" as used herein is meant testing that is related to either the *in vitro* or *in vivo* diagnosis of disease states or biological status in mammals, preferably in humans. By "therapeutics" and "therapeutic/diagnostic combinations" as used herein is respectively meant the treatment or the diagnosis and treatment of disease states or biological status via the *in vivo* administration to mammals, preferably humans, of the antibodies of the present invention. The antibodies of the present invention are especially preferred in the diagnosis and/or treatment of Alzheimer's disease in mammals, preferably humans.

In addition to being functional as direct therapeutic and diagnostic aids, the availability of a family of antibodies which are specific for β -amyloid peptide in the β -sheet conformation enables the development of numerous assay systems for detecting agents which bind to β -amyloid peptide in this specific con-

formation. One such assay system comprises radiolabeling β -amyloid peptide-specific antibodies with a radionuclide such as ^{125}I and measuring displacement of the radiolabeled β -amyloid peptide-specific antibody from solid phase β -amyloid peptide.

Numerous other assay systems are also readily adaptable to detect agents which bind β -amyloid peptide. The aforementioned assay systems are discussed in *Methods in Enzymology*, (J. Langone, and H. Vunakis, eds. 1981), Vol. 73, Part B, the contents of which are herein incorporated by reference. Skilled artisans are directed to Section II of *Methods in Enzymology*, Vol. 73, Part B, *supra*, which discusses labeling of antibodies and antigens, and Section IV, which discusses immunoassay methods.

The antibodies of the present invention are useful in the diagnosis and treatment of mammals suffering from Alzheimer's disease.

In another embodiment, this invention encompasses pharmaceutical formulations for parenteral administration which contain, as the active ingredient, the antibodies described, *supra*. Such formulations are prepared by methods commonly used in pharmaceutical chemistry.

Products for parenteral administration are often formulated and distributed in solid, preferably freeze-dried form, for reconstitution immediately before use. Such formulations are useful compositions of the present invention. Their preparation is well understood by pharmaceutical chemists.

In general, these formulations comprise the active ingredient in combination with a mixture of inorganic salts, to confer isotonicity, as well as dispersing agents such as lactose, to allow the dried preparation to dissolve quickly upon reconstitution. Such formulations are reconstituted for use with highly purified water to a known concentration.

Alternatively, a water soluble form of the antibody can be dissolved in one of the commonly used intravenous fluids and administered by infusion. Such fluids include physiological saline, Ringer's solution or a 5% dextrose solution.

Claims

1. A method for assaying for agents which inhibit the neurotoxicity of β -amyloid peptide which comprises:
 - a. causing a sample of purified β -amyloid peptide to adopt a predominantly β -sheet conformation;
 - b. incubating potential inhibitors of neurotoxicity with the β -amyloid peptide in β -sheet conformation;
 - c. measuring the neurotoxic properties of each β -amyloid peptide/potential inhibitor mixture; and

- d. detecting reduction in the neurotoxicity relative to a control.
2. A method as claimed in Claim 1 wherein said β -amyloid peptide has adopted 80% of its potential β -sheet conformation. 5
3. A method for assaying for agents which inhibit the neurotoxicity of β -amyloid peptide which comprises:
- a. causing a sample of purified β -amyloid peptide to adopt a predominantly non β -sheet conformation; 10
 - b. incubating potential inhibitors of neurotoxicity with the β -amyloid peptide in non β -sheet conformation; 15
 - c. manipulating the β -amyloid peptide in such a way that β -amyloid peptide without the potential inhibitor of neurotoxicity adopts a predominantly β -sheet conformation. 20
 - d. measuring the neurotoxic properties of each β -amyloid peptide/potential inhibitor mixture; and
 - e. detecting reduction in the neurotoxicity relative to a control. 25
4. A method as claimed in Claim 5 wherein said manipulation step is aging of the peptide.
5. A method as claimed in Claim 3 wherein said β -amyloid peptide in predominantly non β -sheet conformation has adopted less than 50% of its potential β -sheet conformation. 30
6. A method for assaying for agents which inhibit the neurotoxicity of β -amyloid peptide which comprises:
- a. allowing a sample of purified β -amyloid peptide to adopt a predominantly β -sheet conformation; 40
 - b. securing the purified β -amyloid peptide in the predominantly β -sheet conformation to a support;
 - c. incubating with the β -amyloid peptide an antibody which diminishes the neurotoxicity of β -amyloid peptide which is in a predominantly β -sheet conformation; 45
 - d. incubating with the β -amyloid peptide/antibody mixture a potential inhibitor of neurotoxicity; 50
 - e. subsequently measuring the amount of unbound antibody in the supernatant;
 - f. determining increase in the amount of unbound antibody in the supernatant relative to a control. 55
7. A conformationally-specific antibody which comprises an antibody having high affinity for β -amyloid peptide only when said β -amyloid peptide is in a predominantly β -sheet conformation.
8. An antibody as claimed in Claim 7 wherein said antibody is a monoclonal antibody.
9. A method of diagnosing Alzheimer's disease in a mammal which comprises administering to a mammal an antibody as claimed in Claim 7.
10. A conformationally-specific antibody which comprises an antibody having high affinity for β -amyloid peptide only when said β -amyloid peptide is in a predominantly random coil or α -helix conformation.



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(54) Pharmaceutical screens and antibodies.

(57) This invention describes a series of assays useful in evaluating the efficacy of agents which inhibit the neurotoxic effects of β -amyloid peptide. These assays employ β -amyloid peptide which is in a predominantly β -sheet conformation. This invention also encompasses antibodies having a specificity for β -amyloid peptide which is predominantly in a β -sheet conformation as well as pharmaceutical formulations containing these antibodies. These antibodies show poor reactivity with β -amyloid peptide which has a great deal of random coil or α -helix secondary structure.

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European Patent
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PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention EP 94 30 1170
shall be considered, for the purposes of subsequent
proceedings, as the European search report

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|---|--|---|--|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl. 8) |
| A | US-A-5 164 295 (KISILEVSKY ROBERT ET AL) 17 November 1992 * column 8, line 11 - line 12; claims * | 1,3,6 | G01N33/68 C07K15/28 |
| A | WO-A-90 05138 (THE CHILDRENS MEDICAL CENTER CORPORATION) 17 May 1990 * claims 1,29 * | 1,3,6 | |
| A | WO-A-92 02248 (THE CHILDRENS MEDICAL CENTER CORPORATION) 20 February 1992 * page 3, line 11 - line 22; claim 13 * | 1,3 | |
| D,A | JOURNAL OF MOLECULAR BIOLOGY, vol.218, 19 October 0, LONDON pages 149 - 163 HILBICH ET AL. 'Aggregation and secondary structure of synthetic amyloid' * page 161, right column * | 1 | |
| A | WO-A-89 06242 (THE MCLEAN HOSPITAL CORPORATION ET AL.) 13 July 1989 * abstract * | 7,8 | |
| | | | TECHNICAL FIELDS SEARCHED (Int. Cl. 8) |
| | | | G01N C07K |
| INCOMPLETE SEARCH | | | |
| <p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely:</p> <p>Claims searched incompletely:</p> <p>Claims not searched:</p> <p>Reasons for the limitation of the search:</p> <p>see sheet C</p> | | | |
| Place of search | Date of completion of the search | Examiner | |
| BERLIN | 23 August 1995 | CEDER O. | |
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EP 0 FORM 1 (01.01.95) (page 1)



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EP 94301170

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claims:
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

☒ LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims 1-8: Assay for agents which inhibit neurotoxicity of beta-amyloid and antibody against beta-amyloid in beta-sheet conformation.
2. Claim 10: Antibody against beta-amyloid in non-beta-sheet conformation.

- ☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respects of which search fees have been paid, namely claims:
- ☒ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims,

namely claims: 1-8

EP 0613 007 A3



EP 94 30 1170

- C -

INCOMPLETE SEARCH

Claims searched completely : 1-8
Claim not searched : 9

Reason : Diagnostic method practised on the human
or animal body (Art. 52(4)).

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| (54) Title: CATIONIZED ANTIBODIES FOR DELIVERY THROUGH THE BLOOD-BRAIN BARRIER (57) Abstract The rate of transcytosis of antibodies across the blood-brain barrier is increased by cationizing the antibodies to provide cationized antibodies having an isoelectric point of between about 8.0 to 11.0. The increased rates of transport across the blood-brain barrier makes such cationized antibodies useful for both neurodiagnostic and neuropharmaceutical purposes. Methods for preparing such cationized antibodies are disclosed. | | |

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CATIONIZED ANTIBODIES FOR
DELIVERY THROUGH THE BLOOD-BRAIN BARRIER

Background of the Invention

5 The present invention relates generally to the use
of antibodies for treatment and diagnosis of neurologi-
cal diseases. More particularly, the present invention
deals with the modification of antibodies so that they
can be delivered through the blood-brain barrier by
10 transcytosis.

This invention was made with Government support
under Grant No.: DK 25744 with the National Institutes
of Health and the University of California. The
Government has certain rights in this invention.

15 Antibodies in general, and especially monoclonal
antibodies, are widely used in diagnostic tests as a
means for detecting the presence of specific antigens.
Enzyme linked immunoassay and radioimmunoassay are
common diagnostic techniques which utilize antibodies
20 and detect antigens in vivo. Antigens may also be
detected in vitro by administering radiolabelled
antibodies to a living subject followed by external
detection of radiolabelled antibody sequestered by a
particular organ bearing the respective antigen.
25 Antibodies have also been used widely in the treatment
of viral infections and other diseases. However, the
use of antibodies in either the treatment or diagnosis
of neurological diseases has been very limited because
most antibodies are not capable of traversing the blood-
30 brain barrier (BBB) and entering the brain.

The vertebrate brain has a unique capillary system which is unlike that in any other organ in the body. The unique capillary system has morphologic characteristics which make up the blood-brain barrier. The blood-brain barrier acts as a system wide cellular membrane which separates the brain interstitial space from the blood. The unique morphologic characteristics of the brain capillaries which make up the blood-brain barrier are: (a) epithelial-like high resistance tight junctions which literally cement all endothelia of brain capillaries together, and (b) scanty pinocytosis or transendothelial channels, which are abundant in endothelia of peripheral organs. Due to the unique characteristics of the blood-brain barrier, antibodies that readily gain access to other tissues in the body are barred from entry into the brain or their rates of entry are very low.

Few strategies have been developed for introducing these antibodies into the brain which otherwise would not cross the blood-brain barrier. The most commonly used strategy involves an invasive procedure where the antibody is delivered directly into the brain. The most common procedure is the implantation of a catheter into the ventricular system to bypass the blood-brain barrier and deliver the antibody directly to the brain. Such a procedure has been used in treating echovirus encephalitis (Erlendsson et al., Successful Reversal of Echovirus Encephalitis in X-linked Hypogammablobulinemia by Intraventricular Administration of Immunoglobulin. 1985. New England Journal of Medicine. Vol. 312, No. 6. pages 351-353).

Although invasive procedures, such as the one described above, for the direct delivery of antibodies to the brain ventricles have experienced some success, they are not entirely satisfactory because they do not deliver the antibodies to the structures deep within the

-3-

brain. Further, the invasive procedures are potentially harmful to the patient. Accordingly, there presently is a need to provide an improved method for delivering antibodies across the blood-brain barrier and into the
5 brain for both diagnostic and therapeutic purposes.

Summary of the Invention

In accordance with the present invention, a method is provided for introducing antibodies directly across
10 the blood-brain barrier by transcytosis. The invention is based upon the discovery that cationized antibodies cross the blood-brain barrier at much higher rates than normal non-cationized antibodies.

The effectiveness of antibodies for both neurodiag-
15 nostic and neuropharmaceutical purposes is increased by cationizing the antibodies to provide cationized antibodies having an isoelectric point (pI) of between about 8.0 to 11.0. These highly basic antibodies cross the blood-brain barrier at rates which are much higher than
20 the transcytosis rates for normal acid and neutral antibodies which typically have isoelectric points in the range of 5 to 6. This provides an effective means for delivering antibodies into the brain by transcytosis rather than by the previously used direct invasive
25 methods.

The cationized antibodies in accordance with the present invention are prepared by treating a given monoclonal or polyclonal antibody with a cationization agent such as hexamethylenediamine. The cationization
30 agent replaces surface carboxyl groups on the antibody with a more basic group, such as a primary amine group in the case of hexamethylenediamine and related amine compounds. The amount of cationization agent and reaction conditions are controlled so that the resulting
35 cationized antibody has an isoelectric point of between about 8.0 to 11.0 and preferably between about 8.0 to

9.0.

As one feature of the present invention, the immunoreactivity of the antibodies is preserved during cationization by first reacting the antibody with an excess of a corresponding antigen to block the immunoreactive sites on the antibody. These blocked immunoreactive sites are unreactive during the subsequent cationization steps. The antigens are then decoupled from the cationized antibodies after the cationization step to thereby reactivate the blocked immunoreactive sites.

The cationization of antibodies in accordance with the present invention is useful whenever it is necessary to introduce an antibody into the brain. Both neurodiagnostic and neurotherapeutic uses for antibodies is contemplated. Particular diagnostic uses include diagnosis of Alzheimer's disease, brain tumors or any other diagnostic use where a labeled or tagged antibody is introduced into the brain for reaction with and detection of specific antigens. Therapeutic uses include treatment of viral infections of the brain or other diseased conditions where introduction of an antibody into the brain is required to treat the disease.

The above discussed and many other features and attendant advantages of the present invention will become apparent as the invention becomes better understood by reference to the following detailed description.

30

Brief Description of the Drawings

Figure 1 is a graph showing the increase in uptake of cationized IgG by brain capillaries at both 4°C and 37°C. The results are expressed as percent uptake of IgG per milligram of IgG.

35

Figure 2 is a graph which also shows the increase

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in uptake of cationized IgG by brain capillaries. The results are expressed as percent uptake of IgG versus amount of brain capillaries.

Figure 3 is a graph showing the uptake of cation-
5 ized IgG plotted versus the concentration of either native IgG or cationized IgG.

Detailed Description of the Invention

The present invention involves the transport of
10 antibodies through the brain capillary wall, ie. the blood-brain barrier (BBB). The nature of the blood-brain barrier and problems associated with transport of peptides and proteins therethrough is set forth in
"Receptor-Mediated Peptide Transport through the Blood-
15 Brain Barrier" (W.M. Pardridge, Endocrine Reviews, Vol. 7, No. 3, August 1986, pages 314 -330), the contents of which is hereby incorporated by reference.

The present invention has wide application to any antibody which is useful in diagnosing or treating brain
20 disorders. Antibodies in general do not readily cross the blood-brain barrier. This is due to the acidic or neutral character of antibodies. It was discovered that the uptake or transport of antibodies into the brain can be greatly increased by cationizing the antibodies to
25 form cationized antibodies having an isoelectric point of between about 8.0 to 11.0.

Antibodies are proteins which have both positive and negative charges with the number of each depending upon the pH of the antibody solution. The pH at which
30 the positive and negative charges are equal is called the "isoelectric point" (pI). Techniques for measuring the pI of a given antibody or protein are well known and generally involve isoelectric focusing according to conventional electrophoresis procedure. As previously
35 mentioned, most antibodies have an isoelectric point of between about 5 to 6.

The relatively low isoelectric point of antibodies is due to the presence of carboxyl groups on the surface of the antibodies. The present invention involves substituting basic groups in place of a sufficient number of surface carboxyl groups to increase the pI of the antibody to between about 8.0 to 11.0. Isoelectric points of between about 8.0 to 9.0 are preferred with isoelectric points of around 8.5 being especially preferred. The degree of cationization should be as high as possible without causing the antibody to form into aggregates. Higher pI's are preferred because the rate of transport of the antibody across the blood-brain barrier increases with increasing pI. However, this must be offset by the increasing possibility of antibody aggregate formation at higher levels of cationization.

Cationization of the antibody can be carried out according to any of the known procedures for displacing surface carboxyl groups on proteins with basic cations. Preferred cationization agents include amine compounds such as hexamethylenediamine and related amine compounds. Hexamethylenediamine is the preferred cationization agent because it is widely available and the techniques for its use in cationizing proteins are well known. The amount of cationizing agent and the conditions for reaction with the antibody can be varied so long as the final cationized antibody has a pI within the above-mentioned range required for blood-brain barrier transport.

The particular antibodies which can be used are virtually unlimited, provided that they have some diagnostic or therapeutic use in connection with the brain. Monoclonal antibodies are preferred because of their increased diagnostic or therapeutic potential. Typical antibodies which can be cationized for blood-brain barrier transcytosis are antibodies to one or more

of the antigenic portions of peptides specific to Alzheimer's disease (Pardridge, W.M. et al., Amyloid Angiopathy of Alzheimer's Disease: Amino Acid Composition and Partial Sequence of a 4,200 - Dalton Peptide
5 Isolated from Cortical Microvessels, Journal of Neurochemistry, 1987, pages 001 - 008). Antibodies to such specific peptides can be tagged with a radioactive tracer or other identifier and then cationized to a pI of 8.5 with hexamethylenediamine. The resulting tagged
10 and cationized antibody can then be administered intravenously to the patient using a suitable pharmaceutically acceptable carrier solution. The tagged and cationized antibody will cross the blood-brain barrier and enter the brain where it will bind to any of
15 the peptides which are unique to Alzheimer's disease. Detection of the bond tagged and cationized antibody which is bound to the specific peptides is then performed by convention of neuroimaging techniques, such as external detection nuclide counting.

20 Other diagnostic antibodies which can be cationized to provide entry into the brain include antibodies for use in detecting various types of brain tumors. For example, monoclonal antibodies to tumor specific proteins such as glial fibrillary acidic protein (GFAP)
25 can be prepared by conventional and well known techniques for monoclonal antibody preparation. Antibodies to human DR antigen and human immunodeficiency virus HIV antigen are other examples.

The resulting monoclonal antibodies are treated
30 with hexamethylenediamine or other cationization agents to increase the pI of the antibody to between about 8.0 to 11.0. The antibody can be tagged with a radioactive tracer prior to or after the cationization process. The resulting tumor specific cationized and tagged antibody
35 is then administered to the patient intravenously for transport across the blood-brain barrier and binding to

any tumor specific antigen. Detection of bound antibody is again accomplished by convention radionuclide scanning techniques.

5 Cationized antibodies for use in treating viral diseases such as AIDS or other disorders of the brain can also be prepared as set forth above. Once an antibody (preferably monoclonal) is prepared for a specific neurotropic virus or other infectious agent, the antibody is cationized to increase its pI to between
10 about 8.0 to 11.0. The antibody is then administered intramuscularly or intravenously to the patient. The antibody is typically administered as a solution of antibody in a suitable pharmaceutical carrier such as saline buffer. The doses of cationized antibody
15 administered for either diagnostic or therapeutic purposes will parallel the dosage levels established for non-cationized antibodies. Typical dosages range from 0.01 mg to 1 mg for diagnostic purposes and from 1 mg to 100 mg for therapeutic purposes.

20 Preferred antibodies include chimeric human antibody molecules designed to have reduced antigenicity, such as those antibodies having mouse antigen-binding domains with human constant region domains. Such chimeric antibodies have been disclosed by S. L. Morrison et al. (Chimeric Human Antibody Molecules: Mouse Antigen-binding Domains with Human Constant Region Domains, Proc. Nat'l. Acad. Sci. USA, November 1984, Vol. 81, pages 6851 - 6855).

Although hexamethylenediamine is the preferred
30 compound for use in cationizing antibodies, other cationizing agents are possible. For example, ethylene diamine, N,N-dimethyl-1,3-propanediamine, or polylysine may be used. Cationization is catalyzed by carboxyl activation using N-ethyl, N¹(3-dimethyl-aminopropyl)
35 carbodiimide hydrochloride (EDAC) using the method described by Hoare and Koshland (A Method for the

Quantitative Modification and Estimation of Carboxylic Acid Groups in Proteins. 1967: J. Biol. Chem. 342:2447-2453).

In order to prevent reductions in the immunore-
5 activity of an antibody during cationization, it is preferred that the antibody be pre-bound to the antigen of interest prior to cationization. This pre-binding with antigen effectively blocks the immunoreactive sites on the antibody and prevents them from being cationized.
10 After cationization is complete and the pI of the antibody has been raised to the desired level between about 8.0 to 11.0, the cationized antibody is then treated to unbind the antigen from the antibody. The unbinding is accomplished according to well known
15 procedures where the antibody-antigen complex is treated with an acid to break the antibody-antigen bond. The antibody is then recovered by column chromatography or other conventional separation and recovery technique.

As an example of practice, bovine IgG was cation-
20 ized and tested against native bovine IgG as follows:

One gram of bovine immunoglobulin G was dissolved in 10 ml of water followed by dialysis at 4°C overnight against water. To this was added slowly 67 ml of 2 M hexamethylenediamine while stirring, and the pH was kept
25 at 7.8. Thirty minutes later, 1 g of EDAC was added and the pH was maintained at 7.8 and the solution was stirred at room temperature for 3-4 hours. The material was then dialyzed against 40 liters of water overnight at 4°C followed by evaporation to dryness the following
30 day.

The cationized antibody and native bovine antibody were then radiolabelled with ^3H -sodium borohydride using standard methods which have been described previously by Pardridge et al. (Absence of Albumin Receptor on Brain
35 Capillaries In Vivo or In Vitro. 1985, Am. J. Physiol. 249:E264-E267; Chimeric Peptides as a Vehicle for

Peptide Pharmaceutical Delivery through the Blood-Brain Barrier. 1987, Biochem. Biophys. Res. Commun. 146:307-315). Bovine brain capillaries were isolated from fresh bovine brain and used as in vitro model system of the blood-brain barrier as reviewed by Pardridge, W.M. (Receptor-Mediated Peptide Transport through the Blood-Brain Barrier. 1986, Endocrine Reviews 7:314-330).

The results of the above tests with bovine IgG are set forth in Figures 1, 2 and 3. In Figure 1, the percent uptake per milligram protein of (^{125}I) cationized IgG or (^{125}I) native IgG at either 37°C or 4°C is plotted versus incubation time. The labeled cationized or native IgG was incubated with isolated bovine brain capillaries, which are used as an in vitro model system of blood-brain barrier transport. The results show that the cationization procedure increases the uptake of the IgG by nearly 50-fold, and that this is partially inhibited by cold temperatures. In Figure 2, the percent uptake of (^{125}I) cationized IgG or (^{125}I) native IgG is plotted versus the amount of bovine brain capillary protein content in micrograms per tube. There is approximately a 25-fold increase in the uptake of the IgG following cationization.

In Figure 3, the percent uptake of (^{125}I) cationized IgG per milligram protein of isolated bovine brain capillary is plotted versus the concentration of unlabeled cationized IgG or native IgG. The data show that the uptake of (^{125}I) cationized IgG is completely independent of concentration of native IgG through three log orders of magnitude in concentration. However, the presence of unlabeled cationized IgG stimulates the uptake in low concentrations and greatly depresses the uptake in high concentrations. The concentration of cationized IgG which causes 50% inhibition is approximately 2.5 mg/ml or approximately 15 μM cationized IgG.

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Further examples of practice are:

- 5 A monoclonal antibody may be prepared against a synthetic peptide corresponding to the 4200 Dalton amyloid peptide of Alzheimer's disease amyloid angiopathy (see Pardridge et al, Amyloid angiopathy of Alzheimer's disease: amino acid composition and partial sequence of a 4,200-Dalton peptide isolated from cortical microvessels, 1987 J. Neurochem 49.) This amyloid is deposited on the brain side of the BBB and, thus, a monoclonal antibody to the amyloid peptide cannot be used as a neuroimaging device unless the monoclonal antibody is transportable through the BBB. The monoclonal antibody to the synthetic amyloid peptide may be cationized using hexamethylenediamine and EDAC, in the presence of saturated concentrations of synthetic amyloid peptide (to protect the active antigen binding sites on the antibody), to an isoelectric point of between 8 - 11. The cationized antibody may then be separated from the antigen by gel filtration in the presence of 0.1 M glycine (pH = 2.5). The high molecular weight peak containing the cationized antibody is then neutralized to pH = 7.4 and is now suitable for radiolabelling using standard radionuclides such as technetium 99m or iodine-I¹³¹.
- 25 A monoclonal antibody to human GFAP may be prepared by isolating GFAP from human autopsy brain using standard techniques or by isolating recombinant human GFAP from either a bacterial or a eukaryotic expressing system. The monoclonal antibody to GFAP may then be cationized using hexamethylenediamine and EDAC in the presence of high concentrations of GFAP, followed by separation of cationized antibody from antigen as described above. The cationized monoclonal antibody to human GFAP may then be radiolabelled with technetium 99m or iodine-I¹³¹ or other conventional radionuclides. The final preparation is a radiolabelled antibody to GFAP
- 30
- 35

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that is transportable through the BBB and may be used as a neuroimaging device for early detection of brain glial tumors.

Another example is the preparation of mouse-human
5 chimeric antibody directed against the human DR-antigen. This mouse-human chimeric antibody may be cationized using hexamethylenediamine and EDAC in the presence of saturating concentrations of recombinant DR-antigen followed by separation of cationized antibody from the
10 free DR-antigen. The cationized human-mouse chimeric monoclonal antibody to the human DR-antigen may then be administered subcutaneously to subjects with demyelinating diseases, such as multiple sclerosis, that have an immune basis, and the pathogenesis of which may be
15 ameliorated by the administration of antibody against the DR-antigen. For example, Sriram and Steinman (Anti I-A Antibody Suppresses Active Encephalomyelitis: Treatment Model for Diseases Linked to IR Genes. 1983, J. Exp. Med. 158:1362-1367) have provided evidence that immune
20 linked demyelinating diseases may be treated by administration of antibody against the class II histocompatibility antigen.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled
25 in the art that the within disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly the present invention is not limited to the specific embodiments as illustrated
30 herein, but is only limited by the following claims.

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WHAT IS CLAIMED IS:

1. In a diagnostic composition comprising one or more antibodies for administration to an individual for neurodiagnostic purposes, said antibodies having a relatively low transfer rate across the blood-brain barrier, wherein the improvement comprises cationizing said antibodies to provide cationized antibodies having an isoelectric point of between about 8.0 and 11.0, said cationized antibodies having increased rates of transfer across said blood-brain barrier.
2. A diagnostic composition according to claim 1, wherein said antibodies are monoclonal antibodies.
3. A diagnostic composition according to claim 2 wherein the isoelectric point of said cationized antibodies is between about 8.0 to 9.0.
4. A diagnostic composition according to claim 1 wherein said antibody is an antibody to an Alzheimer's disease amyloid peptide.
5. A diagnostic composition according to claim 1 wherein said antibody is an antibody to one or more antigens present in GFAP protein.
6. In a neuropharmaceutical composition comprising one or more antibodies for administration to an individual for neurotherapeutic purposes, said antibodies having a relatively low transfer rate across the blood-brain barrier, wherein the improvement comprises cationizing said antibodies to provide cationized antibodies having an isoelectric point of between about 8.0 and 11.0, said cationized antibodies having increased rates of transfer across said blood-brain

10 barrier.

7. A neuropharmaceutical composition according to claim 6 wherein said antibodies are monoclonal antibodies.

8. A neuropharmaceutical composition according to claim 7 wherein the isoelectric point of said cationized antibodies is between about 8.0 to 9.0.

5 9. A neuropharmaceutical composition according to claim 6 wherein said antibody is an antibody to an antigen selected from the group consisting of amyloid peptide of Alzheimer's disease, human GFAP, human DR-antigen, or the human immunodeficiency virus (HIV).

10. A method for preparing an antibody having an increased transfer rate across the blood-brain barrier comprising the step of treating said antibody with an effective amount of a cationization agent to form a
5 cationized antibody having an isoelectric point of between about 8.0 to 11.0.

11. A method according to claim 10 wherein said antibody is a monoclonal antibody.

12. A method according to claim 10 including the additional steps of:

reacting said antibody with an antigen for said antibody to form an antibody having immunoreactive sites
5 blocked by said antigen prior to cationization of said antibody; and

treating the cationized antibody to remove said antigens therefrom to provide a cationized antibody having unblocked immunoreactive sites.

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13. A method according to claim 10 wherein said cationization agent is an amine cationization agent.

14. A method according to claim 13 wherein said amine cationization agent is hexamethylenediamine.

15. A method according to claim 10 wherein cationized antibodies having an isoelectric point of between about 8.0 to 9.0 are formed.

16. A method according to claim 14 wherein cationized antibodies having an isoelectric point of about 8.5 are formed.

17. A method according to claim 10 wherein said antibodies are antibodies for diagnostic uses.

18. A method according to claim 10 wherein said antibodies are antibodies for therapeutic uses.

19. A method according to claim 17 wherein said antibodies are selected from the group consisting of antibodies to Alzheimer's disease amyloid peptide, GFAP protein, DR-antigen and HIV antigen.

20. A method according to claim 18 wherein said antibody is an antibody to an antigen selected from the group consisting of Alzheimer's disease amyloid peptide, GFAP protein, DR-antigen and HIV antigen.

Fig. 2.

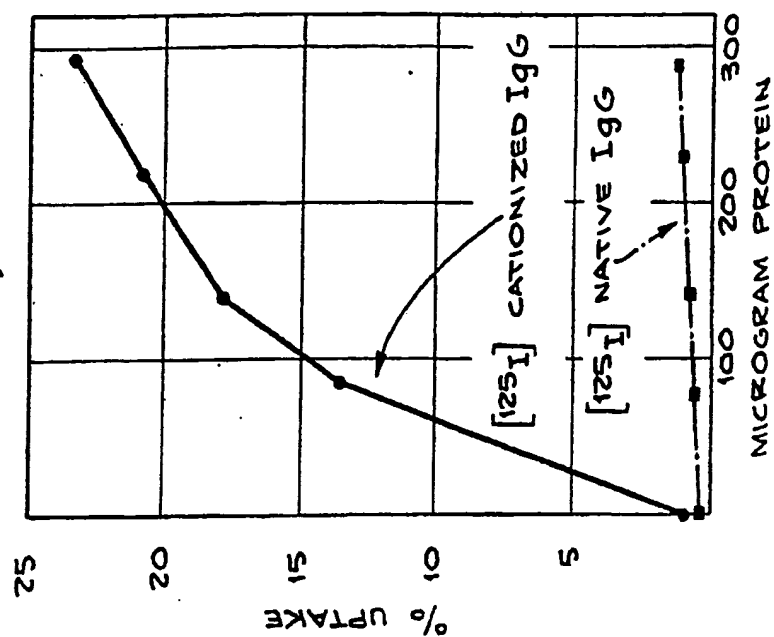
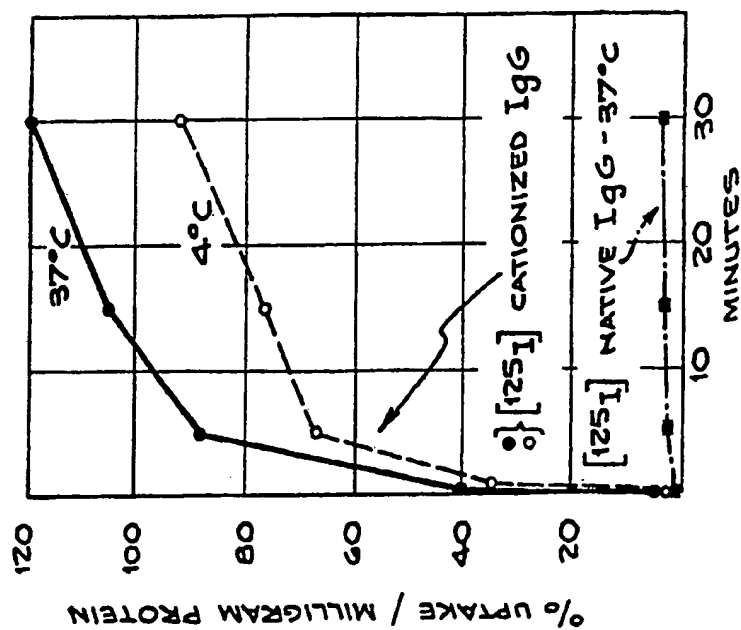
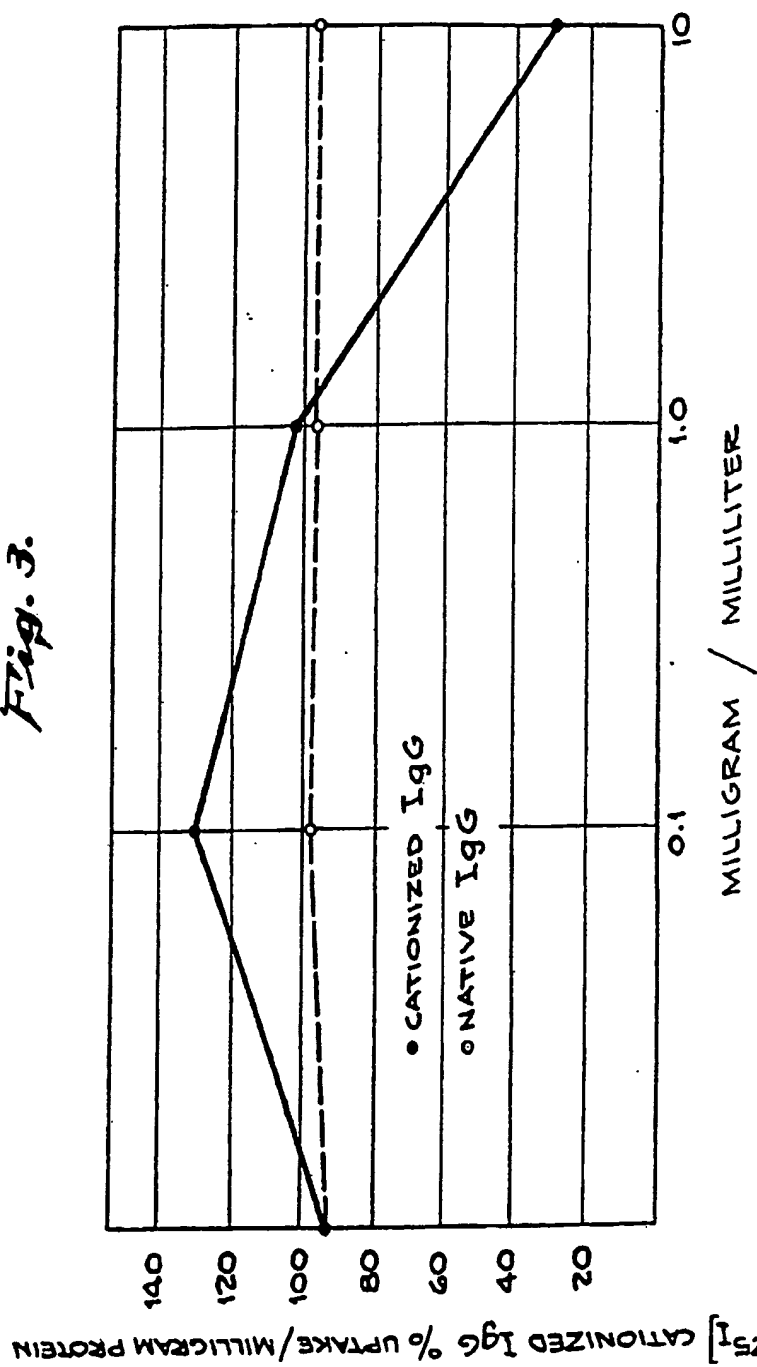


Fig. 1.



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Fig. 3.



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/02760

| | | |
|--|---|--------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| 4 A 61 K 49/00; C 07 K 15/00; A 61 K 39/395; //C 12 P 21/00 | | |
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| III. DOCUMENTS CONSIDERED TO BE RELEVANT* | | |
| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages ** | Relevant to Claim No. ** |
| A | US, A, 4118379 (R. SCHMIDTBERGER) 3 October 1978 see column 1, lines 7-12, line 55 - column 2, line 3; column 2, lines 28-40; claims 1-7, 16 | 1-20 |
| A | The Journal of Immunology, vol. 133, no. 6, December 1984 The American Ass. of Immunologists (US) B.H. Hahn et al.: "A public idiotypic determinant is present on spontaneous cationic IgG antibodies to DNA from mice of unrelated lupus prone strains" pages 3015-3019, see page 3015, abstract, page 3017, left-hand column, paragraph 1 - page 3018, left-hand column, paragraph 1 | 1, 6 |
| A | Chemical Abstracts, vol. 107, 1987, (Columbus, Ohio, US) W.M. Pardridge et al.: "High molecular weight Alzheimer's disease amyloid peptide immunoreactivity in human serum and CSF is an immunoglobulin G", | 1, 4, 6, 9 |
| <p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> | | |
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| | <p>see page 524, abstract no. 37764f & Biochem. Biophys. Res. Commun. 1987, 145(1), 241-8.</p> <p>-----</p> | |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
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US 8802760

SA 24264

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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| <p>(51) International Patent Classification ⁶ : G01N 33/53, A61K 38/00, 31/66, C12N 15/00, C12P 21/06</p> | <p>A1</p> | <p>(11) International Publication Number: WO 96/18900 (43) International Publication Date: 20 June 1996 (20.06.96)</p> |
| <p>(21) International Application Number: PCT/US95/16092 (22) International Filing Date: 13 December 1995 (13.12.95) (30) Priority Data: 08/358,786 16 December 1994 (16.12.94) US (71) Applicant (for all designated States except US): RAMOT-UNIV. AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD. (IL/IL); Tel Aviv University, 32 Haim Levanon Street, Tel Aviv (IL). (71) Applicant (for MW only): SHOSHAN, Herbert, Z. (US/IL); 544 Agur, Makabim (IL). (72) Inventor; and (75) Inventor/Applicant (for US only): SOLOMON, Beka (IL/IL); 120 Hanassi Street, Herzlya Pituah (IL). (74) Agent: KOHN, Kenneth, I.; Kohn & Associates, Suite 410, 30500 Northwestern Highway, Farmington Hills, MI 48334 (US).</p> | <p>(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> | |

(54) Title: PREVENTION OF PROTEIN AGGREGATION

(57) Abstract

A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a native target molecule epitope with a high binding constant and which are non-inhibitory to the biological activity of the target molecule when bound. The method includes the steps of mixing a denatured target molecule with a presumptive anti-aggregation molecule and then determining if the target molecules are prevented from self- or induced-aggregation. The nonaggregated target molecule coupled to the anti-aggregation molecule is then tested for bioactivity.

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PREVENTION OF PROTEIN AGGREGATION**TECHNICAL FIELD**

5 The present invention relates to the use of monoclonal antibodies, genetically engineered antibody fragments and small peptides which mimic antigen binding sites on the antibody for the prevention of protein aggregation *in vivo* and *in vitro*.

10

BACKGROUND OF THE INVENTION

 When proteins are synthesized they generally must fold and assemble into a three dimensional form
15 to be active. Initially, it was thought that proper folding was inherent in the amino acid sequence. Recent work has shown that additional proteins, now referred to as molecular chaperones, are required to mediate the folding process or unregulated aggregation
20 of the polypeptides will occur preventing the formation of functional proteins (Goloubinoff et al., 1989; Welch, 1993). However, despite the existence of chaperones, aggregation of protein still occurs *in vivo* and can contribute to, or cause, various disease
25 states.

 Other factors must contribute to the occurrence of aggregation. These factors can include mutations of naturally occurring chaperones inhibiting function or allowing function with low efficiency
30 (Wetzel, 1994). Further, "pathological" chaperones have been found which have been defined as "a group of unrelated proteins that induce beta-pleated conformation in amyloidogenic polypeptides" (Wisniewski and Frangione, 1992). It would be useful
35 to be able to replace or augment the activity of the chaperones where necessary and to counteract the

activity of pathological chaperones when present.

Additionally, prion diseases have been shown to be caused by, and/or associated with, a conformational protein change wherein a protein normally having an
5 alpha helix conformation is converted to beta strands which are collected into beta sheets (Prusiner, 1995).

Protein aggregation is of major importance in biotechnology for the *in vitro* production of recombinant proteins. *In vitro* aggregation limits the
10 protein stability, solubility and yields in production of recombinant proteins. In cells during production of recombinant proteins, aggregation is a major impediment of recombinant proteins leading to formation of inclusion bodies in the host cells
15 (DeYoung et al, 1993; Wetzel, 1994; Vandenbroeck et al., 1993).

Further, *in vivo* protein aggregation or precipitation is the cause, or an associated pathological symptom, in amyloid diseases such as
20 Down's syndrome, Alzheimer's disease, diabetes and/or cataracts, and in other disorders (DeYoung et al., 1993; Haass and Selkoe, 1993; Wetzel, 1994; Prusiner, 1995).

Several peptides including β -amyloid, have
25 been shown to spontaneously self-associate, or aggregate, into linear, unbranched fibrils in serum or in isotonic saline (Banks and Kastin, 1992; Haass and Selkoe, 1993). At least fifteen different polypeptides are known to be capable of causing *in*
30 *vivo* different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils. Iron, zinc, chromium or aluminum can participate in this aggregation (Bush et al., 1994).

35 Molecular chaperones were initially recognized as stress proteins produced in cells

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requiring repair. In particular, studies of heat shock on enzymes led the way to the discovery of molecular chaperones that function not only during cellular stress but normally to produce properly
5 folded proteins. The heat shock model is still one of the models of choice in studying molecular chaperones (Welch, 1993; Goloubinoff et al., 1989).

Molecular chaperones are a ubiquitous family of proteins that mediate the post-translational
10 folding and assembly of other unrelated proteins into oligomeric structures. They are further defined as molecules whose functions are to prevent the formation of incorrect structures and to disrupt any that form. The chaperones non-covalently bind to the interactive
15 surface of the protein. This binding is reversed under circumstances that favor the formation of the correct structure by folding. Chaperones have not been shown to be specific for only one protein but rather act on families of proteins which have the same
20 stoichiometric requirements, i.e specific domains are recognized by chaperones. This does not provide the specificity required for therapeutic activity.

Further uses and descriptions of molecular chaperones are set forth in PCT published
25 international patent applications 93/11248, 93/13200, 94/08012 and 94/11513 incorporated herein by reference and in particular 94/08012 page 2 line 20 through page 5, line 14.

PCT published international patent
30 application 93/11248 discloses the use of a chaperone in cell culture to promote efficient production of protein in transformed cells by co-expression of the chaperone molecule. This disclosure does not provide specificity as to which proteins are protected except
35 through co-expression with the wanted protein nor does

it provide information on how to use chaperones therapeutically.

PCT published international patent application 93/13200 discloses the use of a chaperone
5 in a purification step for a recombinant protein isolated from a cell culture and also a fusion protein of the chaperone and recombinant protein. This disclosure also does not provide specificity as to which proteins are protected except through co-
10 expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/08012 discloses the use of a chaperone in cell culture to promote increased secretion of an
15 overexpressed gene product in a host cell. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/11513 discloses the use of a vector containing a molecular chaperone for treating neoplasms. This disclosure does not provide specificity as to which proteins are protected except
25 through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically to treat diseases or syndromes which involve protein aggregation.

In each of the aforementioned publications,
30 the chaperones did not bind to native proteins and did not redissolve aggregated proteins.

Recent reports suggest that monoclonal antibodies (mAb) can have chaperone-like activity. The feasibility of using monoclonal antibodies to
35 assist in the in vitro refolding process of guanidine-denatured S-protein was reported recently (Carlson and

Yarmush, 1992). Previously, Blond and Goldberg (1987) used mAbs as a tool in the identification and characterization of folding steps that involve the appearance of local native-like structures in B₁ subunit of tryptophan-synthase. Since the mAb is epitope specific, the use of a mAb provides more specificity than molecular chaperones. Monoclonal antibodies can be sought and engineered (Haber, 1992) that bind to the particular epitope in the protein of interest that is involved in the folding process.

The main difference between mAbs and molecular chaperones is that the latter does not bind to native proteins and is capable of interacting with many different polypeptide chains without exhibiting an apparent sequence preference (Goloubinof et al., 1989). Moreover, chaperones suppress aggregation but do not redissolve aggregate proteins already present. Similar behavior was recently reported for α -crystallin which, similar to other chaperones, does not react with active proteins, but forms a stable complex with denaturing or partially unfolded proteins, stabilizing against further aggregation (Rao et al., 1994).

Aggregated amyloid β -protein (β A4) is a major constituent of the abnormal extracellular amyloid plaque that characterizes the brains of victims of Alzheimer's disease (AD) (Haass and Selkoe, 1993). *In vitro* studies have shown that some of the metal ions found in biological systems, i.e. Fe, Al and Zn, can accelerate the aggregation process dramatically. The presence of "pathological" chaperones (Wisniewski and Frangione, 1992) and the above listed metals (Mantyh et al., 1993; Fraser et al., 1993) as proposed risk factors in Alzheimer's disease, favor β -amyloid cascade aggregation. If the interaction between the metal ion and the β -amyloid can be interrupted or prevented, then metal-induced

aggregation can be reduced or eliminated. However, just binding a mAb at this site might prevent the metal-induced aggregation but would not allow normal functioning of the protein.

5 It would therefore be useful to develop the appropriate mAb with chaperone-like characteristics directed to the appropriate epitope on the β -amyloid molecule in order to prevent the accelerated metal-induced aggregation.

10 In prion diseases it has been shown that the pathologic "scrapie prion protein" propagates itself by contacting normal prion protein and causing them to unfold and flip from their usual conformation to the scrapie prion protein state, a beta-pleated sheet.

15 This initiates a cascade effect. It would therefore be useful to be able to interrupt the "scrapie-prion" cascade.

 Further, it would be particularly useful to be able to develop a mAb as needed that prevents the aggregation of proteins/enzymes *in vivo* but that still allows the proteins/enzymes to function even when bound to the mAb.

20 With the advent of recombinant DNA methods for the production of proteins in bacteria methods are needed to prevent aggregation during production and harvesting. Chaperones have been shown to suppress aggregation during the folding of a number of protein *in vitro* and co-expression of chaperones can also suppress formation of inclusion bodies by some foreign proteins *in vivo* as described herein. However, despite the use of chaperones, protein aggregation can still occur during production and harvesting.

30 Still further, it is not always possible to isolate the appropriate chaperone for preventing aggregation of a molecule and to utilize it as a therapeutic. The availability of engineering and

selecting mAbs and delivery systems for mAb makes it useful to develop specific mAb to serve as therapeutic chaperones.

5 **SUMMARY OF THE INVENTION AND ADVANTAGES**

According to the present invention, a method is provided of selecting an anti-aggregation molecule such as a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics
10 the binding site of an antibody. These anti-aggregation molecules are able to bind to a native target molecule epitope with a high binding constant and are selected to be non-inhibitory to biological activity of the target molecule.

15 The present invention further provides a method of treating a protein aggregation disease by creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human form of the anti-aggregation molecule that
20 binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.

The present invention also provides an expression vector comprising nucleic acid including a
25 sequence which encodes in expressible form the human form of the anti-aggregation molecule that binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.

30

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed
35 description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a bar graph of the temperature-dependence of enzymic activity of Carboxypeptidase A (CPA); the residual enzymic activity of CPA after one hour incubation at increasing temperatures was measured using esterase substrate;

FIGURE 2 is a bar graph of the time course of denaturation of Carboxypeptidase A after exposure at 50°C; the residual esterase (□) and peptidase (cross-hatch) enzymic activity of CPA was measured at two intervals of incubation at 50°C; the amount of residual soluble enzyme was determined by sandwich ELISA (■);

FIGURE 3 is a bar graph of the enzymic activity of Carboxypeptidase A retained after exposure to 50°C for one hour in the presence of monoclonal antibody CP₁₀; the immunocomplexation of CPA with increasing amounts of CP₁₀ was performed before exposure at 50°C for one hour; the residual peptidase (■) and esterase (□) enzymic activity of CPA was measured;

FIGURE 4 is a bar graph of the effect of epitope location on the maintenance of the enzymic activity of heat-exposed Carboxypeptidase A; increasing amounts of monoclonal antibodies CP₁₀ (cross-hatch) and CP₉ (□) and unrelated IgG (■) were added to CPA before exposure to 50°C for one hour and esterase enzymic activity was measured;

FIGURE 5 is a bar graph of the prevention of aggregation of Carboxypeptidase A by monoclonal antibody CP₁₀; aggregation of CPA, in the presence (double cross-hatch bars) and in the absence (single cross-hatch bars) of antibodies, was followed by

determination of amount of mAb bound to coated CPA in a competitive ELISA; the absorbance at 495 nm obtained in the absence of added soluble CPA was set at 100% for bound antibody; the soluble CPA, before heat

- 5 exposure, competes with the coated CPA for antibody binding, leading to decrease in amount of antibody bound (60%) (diagonal lines);

- FIGURE 6 is a bar graph showing thermal
10 aggregation of Carboxypeptidase A and its suppression by monoclonal antibodies CP₁₀ and CP₉; aggregation of Carboxypeptidase A after exposure at 50°C for one hour in the absence (□) of monoclonal antibodies and in the presence of CP₁₀ (cross-hatch) and CP₉ (■) was followed
15 by determination of amount of antibody bound by sandwich ELISA; maximum binding (100%) was considered the amount of antibody bound to CPA before exposure to aggregation conditions;

- 20 FIGURE 7 is a pair of graphs (A and B) showing aggregation of β -amyloid (1-40) in the absence (cross-hatch) and in the presence (□) of monoclonal antibodies AMY-33 (A) and 6F/3D (B) followed by ELISA; (1) β -amyloid alone, (2) β -amyloid + 50 mM heparan
25 sulfate, (3) β -amyloid + 10^{-3} M AlCl₃; (4) β -amyloid + 10^{-3} M ZnCl₂;

- FIGURE 8 is a pair of graphs (A and B) showing a Thioflavin T based fluorimetric assay of β -
30 amyloid aggregation (■) in the presence of mAb AMY-33 (□) and an unrelated antibody (cross-hatch) with (A) an emission spectra of Thioflavin T bound to fibrillar β -amyloid peptide (upper curve) and in the presence of antibody AMY-33 the immunocomplex with β A4 (lower
35 curve) and (B) shows an increase in Thioflavin T fluorescence bound to β -amyloid peptide (■) as a

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function of incubation time of the peptide as measured after three hours, three days and seven days wherein immunocomplexation with mAb AMY-33 prevents the increase in Thioflavin T fluorescence (\square), while
5 unrelated antibody (cross-hatch) did not interfere with its fluorescence; and

FIGURE 9 is a bar graph showing aggregation in the presence of various monoclonal antibodies to β -
10 amyloid peptide in the absence of soluble β -amyloid (100%, maximal binding) and calculated by comparison with the binding of the same monoclonal antibodies to the residual soluble β -amyloid peptide remaining after incubation in the absence of the respective antibody.

15

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a method of selecting monoclonal antibodies, genetically
20 engineered antibody fragments and small peptides which mimic binding sites of the antibodies that prevent aggregation of a native target molecule, which may aggregate and is in general a protein, and yet do not inhibit bioactivity when bound to the target molecule.
25 These anti-aggregation molecules with chaperone-like activity are able to bind to a native target molecule epitope with a high binding constant and yet are selected to be non-inhibitory to the biological activity of the target molecule when bound.

30 The method can include culturing an appropriate host cell transformed with DNA encoding the target molecule. The host cell chosen will express the target molecule in aggregated form. Examples of such cells are set forth in PCT published
35 international patent application 93/11248, 93/13200 and 94/08012. Alternatively, the appropriate

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recombinant target molecule can be purchased or a naturally occurring molecule can be isolated or purchased.

The expressed target molecule is recovered
5 and denatured thereby also deaggregating the molecules if they are aggregated. The denatured target molecule is mixed with the presumptive anti-aggregation molecule such as a monoclonal antibody, genetically engineered antibody fragment or small peptide which
10 mimics an antibody binding site generally as set forth in PCT pending application 93/13200 and under conditions which allow for renaturing and for self-aggregation such as temperature, pH or interaction with other aggregation-inducing agents. Mixtures
15 which produce non-aggregated target molecules are selected. It is then determined if the nonaggregated target molecules are bioactive even in the presence of, and bound to, the presumptive anti-aggregation molecule.

20 In addition, the anti-aggregation molecule is screened for its ability to dissolve already aggregated proteins. The aggregated proteins are mixed with the anti-aggregation molecules under physiological conditions. Mixtures with non-
25 aggregated molecules are selected. It is then determined if the nonaggregated target molecules are bioactive even in the presence of, and bound to, the presumptive anti-aggregation molecule.

The antibodies, or peptide mimicking the
30 binding site, bind to an epitope on the target molecule which is a region responsible for folding or aggregation. In addition the anti-aggregation molecule is selected only if it does not show immune cross reactivity with other proteins with proximity to
35 the target molecules under the same conditions employed in the bioactivity tests; that is, molecules

which are found in the cell near the target molecule or molecules with sequences similar to the target molecules.

After the identification of the anti-
5 aggregation molecules has been completed, it is possible in one embodiment to utilize two or more to prevent or reverse aggregation. They can be used concurrently to increase their chaperone-like effect, if their respective target epitopes are not
10 overlapping and if, in binding to the target molecule, they do not interfere with each other.

Bioactivity is tested as is appropriate for the target molecule. For example, enzymatic activity of the target molecule for its substrate can be
15 measured. Assays which measure *in vitro* enzymatic bioactivity are well known to those skilled in the art.

In one embodiment of the method, the target molecule is β -amyloid and the monoclonal antibody is
20 an anti- β -amyloid monoclonal. Alternatively, a genetically engineered antibody fragment as described hereinbelow can be used or a small peptide which mimics the antigen binding site on the target molecule can be used. The antigen binding site of an antibody
25 can be determined as is known in the art as for example from x-ray crystallography, DNA sequences or the like.

The method has also been demonstrated with carboxypeptidase A as set forth in the Examples
30 hereinbelow.

Other peptides or proteins with evidence of self aggregation can also be used in the present invention such as amylin (Young et al., 1994); bombesin, caerulein, cholecystokinin octapeptide, elodeisin, gastrin-related pentapeptide, gastrin
35 tetrapeptide, somatostatin (reduced), substance P; and

peptide, luteinizing hormone releasing hormone, somatostatin N-Tyr (Banks and Kastin, 1992), and prion protein (PrP).

Once an appropriate monoclonal antibody with
5 chaperone-like activity is found or engineered or a peptide with the appropriate configuration, the present invention provides for its use therapeutically to prevent or reduce protein aggregation *in vivo*. In an embodiment, the prevention of β -amyloid aggregation
10 or prion-associated protein aggregation is undertaken.

A method of treating a protein aggregation disease intracellularly includes the steps of preparing (Haber, 1992; Harlow & Lane, 1988) or selecting an anti-aggregation molecule as set forth
15 herein, such as a monoclonal antibody, genetically engineered monoclonal antibody fragment or peptide that mimics the binding site of an antibody, that binds to an aggregating protein which is the cause of a disease and which prevents aggregation and yet
20 allows the protein to be bioactive even while bound. This molecule can be referred to as an anti-aggregation molecule with chaperone-like activity. An expression vector is created comprising nucleic acid including a sequence which encodes in expressible form
25 the anti-aggregation molecule. The expression vector is then delivered to the patient.

In an embodiment a human monoclonal antibody that binds to an aggregating protein and which prevents aggregation is utilized. In a further
30 embodiment the monoclonal antibody (mAb) is an anti- β -amyloid and is selected from the group consisting of AMY-33 which recognizes an epitope spanning amino acids 1-28 of β -amyloid, mAbs 6C6 and 10D5, which recognize an epitope spanning the amino acid residues
35 1-16 of β -amyloid peptide, and mAbs 2H3 and 1C2,

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directed to the regions comprising peptides 1-12 and 13-28, respectively.

Work by Dueñas et al. (1994) and Marasco et al. (1993) have shown that single chain monoclonal antibodies are efficient for intracellular expression in eukaryotic cells. The single chain monoclonal antibody is composed of an immunoglobulin heavy chain leader sequence and heavy and light chain variable regions that are joined by an interchain linker. The molecule is small, approximately 28 kDa with high-affinity ligand-binding capability and minimal assembly requirement. The antibody can be directed to the relevant cellular compartment using classical intracellular-trafficking signals. Marasco et al. (1993) have shown that such antibodies are not toxic to the cells and function when expressed in the cell.

The production of expression vectors is well known to those skilled in the art. In a preferred embodiment, the expression vector is constructed using the methodology as set forth by Dueñas et al. (1994), PCT pending application 94/11513. Methods not explicitly set forth are performed as generally set forth in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989).

Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors.

Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain
5 elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The expression vector can be a virus.

10 Further the virus can be an RNA virus such as a disabled retro virus or a retroviral shuttle vector. The expression vector can also be vaccinia virus or an adenovirus. The expression vector can also be a plasmid. In a preferred embodiment wherein β -amyloid
15 is the targeted molecule the expression vector is selected that is known to target the central nervous system.

In the present invention, the expression vector for use as a therapeutic agent comprises a
20 nucleic acid including at least one sequence which encodes in expressible form an anti-aggregation molecule, which molecule binds to an aggregating protein that is the cause of a disease and which prevents aggregation but does not interfere with
25 bioactivity. In an embodiment the expression vector includes the sequence for a human monoclonal antibody that is an anti- β -amyloid monoclonal antibody with heparan-like characteristics. In a further preferred embodiment, the expression vector includes the
30 sequence for the single chain monoclonal antibody of the above anti- β -amyloid mAb.

A specific example of a DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector
35 expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an

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expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor. This vector, as well as others that exhibit similar desired functions, can be used to
5 treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its
10 therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus such as antibiotic sensitivity. Negative selection is therefore a means by which infection can be controlled
15 because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur. Features that
20 limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are
25 useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a
30 single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of
35 infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be

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produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

- 5 As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral
- 10 vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be
- 15 targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the nervous system are to be treated,
- 20 then a viral vector that is specific for neural cells such as neurons, oligodendroglia and the like and their precursors, preferably for the specific type of hematopoietic cell, would be used.

- Retroviral vectors can be constructed to
- 25 function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new
- 30 viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired
- 35 recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to

destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through
5 subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology as well as many
10 being commercially available.

The expression vector containing the sequence for the anti-aggregation molecule may be administered to mammals, including humans, by any route appropriate to the condition being treated and
15 in several ways. Suitable routes include oral, rectal, nasal, topical, vaginal and parenteral. It will be appreciated that the preferred route may vary with, for example, the condition of the recipient and the type of treatment envisaged.

20 If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more
25 effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into spinal fluid can also be used as a mode of administration, especially in the case of neuro-
30 degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection. Alternatively, the method as set forth by Tuomanen et al. (1993) can be used.

35 The vectors can be introduced into cells or tissues by any one of a variety of known methods

within the art. Such methods can be found described in Sambrook et al. and Ausubel et al., and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods as indicated herein. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types in vivo or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

An alternate mode of administration of the vector can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into

recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The expression vector of the present invention may be administered to the patient alone or in combination with liposomes or other delivery molecules. The expression vector is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve in the treated patients a reduction in protein aggregation and may also include but is not limited to improved survival rate, more rapid recovery, or improvement or elimination of symptoms and are selected as appropriate measures by those skilled in the art.

While it is possible for the expression vector containing the sequence for the anti-aggregation molecule to be administered alone, it is preferable to present it as a pharmaceutical formulation. The formulations of the present invention comprise at least one active ingredient: the monoclonal antibody or expression vector together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipients. The carriers must also be selected so as not to interfere with the activity of the active ingredient.

Further the present invention also provides a method of improving solubility and yields in

production of recombinant proteins, particularly in blocking the formation of inclusion bodies in the host cells for the *in vitro* production of recombinant proteins. The present invention provides an anti-
5 aggregation molecule that suppresses the kinetics of aggregate formation while still encouraging formation of native protein structure, and favors the desired folding reaction thereby improving yields of the recombinant product. A genetically engineered
10 antibody fragment as described herein can be used or a small peptide which mimics the antigen binding site on the target molecule can be used.

In a preferred embodiment a single-chain antibody (single-chain variable region; SCFVS) in
15 which the heavy and light chain variable domains is engineered as a single polypeptide (Dueñas et al., 1994; Marasco et al., 1993) and delivered to the cells, either directly by methods described herein above or in an appropriate expression vector as
20 described herein. Utilizing an expression vector, the expression of the antibody and of the desired gene product, in general an overexpressed recombinant product, are effected by introducing the either two vectors one encoding the gene for the recombinant
25 produce and one for the antibody or a vector containing both into the host cell. The result is a small, approximately 28 KDa molecule with high-affinity ligand-binding capability and minimal assembly requirement that is co-synthesized with the
30 recombinant protein product in the host cell. The antibody can be directed to the relevant cellular compartment using classical intracellular-trafficking signals. Functional studies have illustrated that single chain antibodies are able to fold and assemble
35 correctly in the cytoplasm. The co-expression of the antibody and the recombinant product increases

intracellular production of the fusion proteins and prevents the formation of the inclusion bodies and/or facilitates extracellular protein secretion. The fusion proteins obtained, in addition to better
5 solubility and yield, exhibit increased thermal and proteolytic resistance.

The availability of monoclonal antibodies which bind to a specific antigen at distinct and well defined sites has led to a better understanding of the
10 effects of highly specific enzyme-antibody interactions on the enzyme behavior. By appropriate selection it has been possible to isolate those antibodies that are non-inhibitory to biological activity of the enzyme and bind at "strategic
15 locations" on the antigen molecule, resulting in a considerable stabilization effect of the enzyme conformation. Moreover, such monoclonal antibodies, by selection using the present invention, prove to have a chaperone-like activity leading to a
20 considerable refolding effect on the enzyme which was already partially heat denatured. In addition, the use of engineered monoclonal antibodies and their fragments, as well as peptides which mimic the binding site for the antigen on the antibody can be used in
25 the present invention.

In the model test system shown in Example 1 herein below, Carboxypeptidase A (CPA) shows a decrease in solubility with an increase in temperature, accompanied by loss of enzymic activity
30 and conformational changes leading to its aggregation. In the Example, the suppression of enzyme aggregation via its interaction with two monoclonal antibodies raised against native protein was investigated. ELISA measurements and determination of residual enzymic
35 activity, as a probe of the native structure, were used to monitor the protein aggregation process. The

studied monoclonal antibodies are non-inhibitory to the biological activity of the antigen or target molecule, bind on the strategic position on the molecule and proved to have a chaperone-like activity

5 in the prevention of protein aggregation. The antibodies effect on the inhibition of aggregation was found to be related to the location of the antigenic site of each antibody. Based on the experimental data, the formation of the immunocomplexes will

10 provide a general and convenient method for suppression of aggregation and stabilization of the target molecules without affecting the biological properties of the given target molecule. The present invention uses genetically engineered antibodies

15 obtained from such selected antibodies as protecting agents of *in vivo* aggregation of their antigen, leading to production of a soluble and stabilized protein.

Protein aggregation is of major importance

20 that extends into mechanisms of human diseases and fundamental aspects of protein folding, expression and function. Data in the literature (De Young et al., 1993; Wetzel, 1994; Wetzel, 1991) suggests that aggregation is non-specific in the sense that addition

25 of other proteins can influence the extent of aggregation of a certain protein. However, the specificity can be related to a particular residue or group of residues which play a special role in the folding-related aggregation of a polypeptide (Silen

30 and Agard, 1989; Zhu et al. 1989; Winter et al., 1994; Brems 1988). The identification of such classes of sequences that play a role in the folding-unfolding and/or solubilization-aggregation provides the basis of the present invention for the prevention of

35 aggregation.

Stabilization procedures based on protein-protein recognition processes, fundamental to biology, have been previously investigated (Chothia and Janin, 1975; Jaenicke, 1991). Introduction of molecular
5 chaperones which enable folding and stabilization of unrelated proteins appears to be tailored to prevent misfolding and aggregation at an early stage during folding. However, the central problem remaining in in vivo folding is how to efficiently prevent aggregation
10 without blocking the forward pathway of correct folding and biological activity of the native state (Ellis et al. 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993).

The availability of monoclonal antibodies
15 (mAbs) led to a better understanding of the effect of highly specific antigen-antibody interactions on the antigen or target molecule behavior. The complementary conformation between the interacting regions of the antibody with its antigen confers the
20 high specificity and stability to the immunocomplex formed (Goldberg, 1991). Properly selected monoclonal antibodies, unlike the ubiquitous nature of the chaperones, bind to a specific antigen at a distinct and preselected antigenic site without interfering in
25 the biological activity of the antigen and assist in antigen refolding (Blond and Goldberg, 1987; Carslon and Yarmush, 1992; Solomon and Schwartz, 1995).

The present invention utilized the effect of immunocomplexation in the suppression of antigen
30 aggregation using as a model system the interaction of Carboxypeptidase A (CPA) and its monoclonal antibodies. CPA occupies a prominent position in the literature of metalloenzymes, being a well-characterized zinc exopeptidase that exhibits both
35 peptidase and esterase activity (Vallee and Galles, 1984). A large number of mAbs were prepared by the

applicant towards native enzymes (Solomon et al. 1984) and their properties were investigated. Some of these antibodies bind to the enzyme with a relatively high binding constant, remote from its active site and
5 assist in refolding of already heat denatured enzyme (Solomon and Schwartz, 1995). ELISA measurements and determination of residual enzymic activity as a probe of native structure are used to monitor the effect of two different mAbs, namely CP₁₀ and CP, on the
10 inhibition of CPA aggregation.

Further, as shown in Examples 2 and 3 herein below, in suppressing β -amyloid aggregation, the monoclonal antibodies AMY-33 and 6F/3D, which recognized different epitopes of the β -amyloid peptide
15 chain, exhibited a selective chaperone-like activity. The immunocomplex of mAb AMY-33 + β A4 not only prevented self-aggregation of β -amyloid peptide but also the aggregation that was induced in the presence of heparan sulfate, which is thought to affect only
20 the aggregation of preexisting amyloid fibers (Talafoos et al., 1994). The inhibitory effect was related to the localization of the antibody-binding sites and to the nature of the aggregating agents. The results of negative-staining electron microscopy
25 revealed that even at low concentrations of mAb AMY-33 only amorphous aggregates are formed. The ELISA measurements indicated that increasing the concentration of mAb AMY-33 to equimolar antigen/antibody ratios maintained β -amyloid peptide
30 solubility. The diffuse and amorphous conglomerates of A β deposits that were not detectable by thioflavin T fluorimetry or Congo Red staining are not supposed to be associated with neuritic pathology (Levine, 1993).

35 Because β -amyloid peptide has been shown to be physiologically produced in a soluble form in

normal individuals (Seubert et al., 1992; Shoji et al., 1992), the aggregation of soluble β -amyloid peptide into insoluble amyloid fibrils is believed to be a crucial step in the pathogenesis of Alzheimer's disease. Therefore, to reduce or eliminate the extent of pathological protein depositions in the brain, much effort has been focused on developing potent and selective inhibitors of β -amyloid aggregation (Synder et al., 1994; Tomiyama et al. 1994; Schwarzman et al., 1994). Preparing mAbs against "aggregating epitopes," identified as sequences related to the sites where protein aggregation is initiated, thereby provides a tool for preventing the phenomenon of protein aggregation. Applicant has shown that appropriate mAbs interact at strategic protein-folding-initiation sites, leading to a considerable refolding effect of the already clustered epitopes.

The mAb AMY-33 did not exhibit a similar inhibitory effect on metal-induced amyloid aggregation. The slight interference with Zn^{2+} -induced β -amyloid peptide aggregation that occurred using mAb 6F/3D may be due to the partial solvation effect of already aggregated β -peptide.

In experiments with additional monoclonal antibodies, mAbs 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β -amyloid peptide, inhibited the formation of β -amyloid by 90% when compared with aggregation occurring in the absence of the respective antibodies. mAb AMY-33, which recognizes another epitope located within residues 1-28, affected the self-aggregation of β -peptide to a lower extent of approximately 40% in the same set of experiments. The antibodies, 2H3 and 1C2, directed to the regions comprising peptides 1-12 and 13-28, respectively, had a considerably lower effect on *in vitro* amyloid formation.

The above discussion provides a factual basis for the use of monoclonal antibodies and genetically engineered antibody fragments as therapeutics for the prevention of protein aggregation. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

10

METHODS AND REAGENTS

Carboxypeptidase A (CPA)

CPA was obtained as an aqueous crystalline suspension (Sigma Chemical Co., St. Louis, MO). The crystals were washed with double-distilled water, centrifuged, and dissolved in 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5. Insoluble material was removed by centrifugation. The enzyme concentration was derived from the absorbance at 278 nm.

20

Determination of CPA Enzymatic Activity

The enzymatic activities of CPA and its immunocomplexes were determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine as peptidase substrate or hippuryl-DL- β -phenyllactic acid as esterase substrate in 0.5 M NaCl/0.05 M Tris-HCl, pH 7.5, (Solomon et al, 1989).

Amyloid

Amyloid peptides, A β 1-40 (Cat. No. A-5813) and A β 1-28 (Cat. No. A-1084) corresponding to amino acids 1-40 and 1-28 of A β respectively, were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Amyloid solutions were prepared by dissolving the peptides in water at concentration of

10 mg/ml. The stock solution was stored in aliquots at -20°C.

Aggregating agents

- 5 Heparan sulfate (Cat. No. H 5393) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Stock solutions of metal chlorides were made up from dry salts at concentration of 1mM in TRIS pH 7.4.

10 Monoclonal Antibody Production

- In general, monoclonal antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce monoclonals by standard production technology well known to those skilled in the art as further described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, Milstein (1980) and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.M. Freeman and Co., 1992. Briefly, mouse monoclonal antibodies are prepared by hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

- The harvested monoclonal antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. For a general discussion of

conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982. The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β -galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Alternatively, commercially available antibodies can be used. α -Human β -amyloid 6F/3D was obtained from ACCURATE Chemical and Scientific Corp. (Westbury, NY, USA). mAb AMY 33 was purchased from ZYMED San Francisco, CA, USA. A polyclonal, affinity purified rabbit IgG obtained against the synthetic Alzheimer β -amyloid (Cat. No. 1381431) was purchased from Boehringer-Mannheim, GmbH, Germany. Another four monoclonal antibodies called 6C6 and 10D5, raised against peptide 1-28 of β -amyloid, 2H3 and 1C2 raised against peptides 1-12 and 13-28, respectively, were generously provided by Dr. D. Schenk, Athena Neuroscience, San Francisco, CA.

Purification and characterization of anti-CPA mAbs

The monoclonal antibodies, CP-10, CP-9, which interact with CPA at high binding constants, were selected for further study. The preparation and characterization of the monoclonal antibodies CP₁₀ and CP₉ (chosen for the present study) were previously described (Solomon et al., 1989; Solomon and Balas, 1991).

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These antibodies were isolated and purified by affinity chromatography on protein A-Sepharose from the corresponding ascites fluids according to Harlow and Lane.

5

Protocol for determining effect of monoclonal antibody binding on CPA activity

CPA (1 mg/ml) was incubated at 50°C in the absence and in the presence of increasing amounts of mAbs CP₁₀ and CP, (100 µl in PBS) ranged between 0-2 molar ratio antibody/CPA. The enzymic activities of the immunocomplexes formed were measured as described herein above. Data related in percentage, 100% being considered the enzymic activity of CPA before denaturation.

15

ELISA Tests for CPA Studies

The antigen-coating solutions (100 µl containing native CPA (10-25 µl/ml) in PBS, pH 7.4, were incubated overnight at 4°C in a polystyrene ELISA plate (Costar, Cambridge, MA). Diluted ascites fluid (0.1 ml) containing the desired mAb (1:2000 to 1:18,000 v/v in PBS) was added and incubated at 37°C for 1 hour. The amount of bound mAb was determined with β-galactosidase-linked F(ab)₂ fragments of sheep anti-mouse IgG (Amersham International, UK).

25

The quantitation of the amount of aggregated CPA during denaturation at 50°C was determined by competitive and sandwich ELISA, as follows:

30

Competitive ELISA assays for CPA Studies

CPA (10 µl/ml of PBS) was adsorbed onto ELISA plates overnight at 4°C, the remaining active groups on the plate being blocked with non-fat milk. To the soluble CPA (200 ng in 10 µl PBS), incubated for one hour at 50°C, the mAb CP₁₀ (molar ratio 1:1

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Ab/CPA) was added and allowed to interact with the remaining soluble CPA for one hour at 37°C. In parallel, the mAb was added to the CPA solutions before exposure at 50°C for one hour. After

5 incubation, the CPA preparations were removed by centrifugation at 15,000 rpm for 15 minutes and applied on the ELISA plates coated with CPA. The antibody which did not bind to soluble CPA in the reaction mixture will bind to the coated CPA; the

10 amount of antibody bound to the coated antigen will be conversely proportional to the extent of CPA aggregation and determined using α -mouse antibodies labeled with horseradish peroxidase (HRP). The color developed by HRP (O-phenylenediamine (OPD) as

15 substrate) was measured at OD₄₉₅ using an ELISA plate reader. The amount of antibody bound on the coated CPA in the absence of soluble CPA was considered as 100%.

20 Sandwich ELISA for CPA Studies

The ELISA plates were coated with rabbit polyclonal antibodies raised against CPA (1 μ l/well) by incubation at 37°C for two hours. The residual active groups were blocked by non-fat milk. Soluble CPA (200

25 ng in 10 μ l PBS) was exposed to 50°C for one hour and the aggregated CPA was removed by centrifugation at 15,000 g for 15 minutes. The residual soluble CPA was incubated for another one hour at 37°C with mAb CP₁₀ and mAb CP₉ at various molar ratio antibody/antigen.

30 In another set of experiments, the mAbs were added to the reaction mixtures before incubation at 50°C and then exposed for one hour at 50°C. After the incubation period, all the immunocomplexed CPA preparations were centrifuged and added to the ELISA

35 plate, previously coated with polyclonal CPA antibodies, for 12 hours at 4°C. The amount of mAb

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bound, determined as described above, will be proportional to the amount of soluble CPA which remained after exposure to aggregation conditions. The results are presented in percentages, 100% being the maximal absorbance obtained before CPA heat treatment.

All data presented are the mean of triplicate determinations. The standard deviation of the intra-assay and interassays were less than 5% in all cases.

Amyloid ELISA Assays

The ELISA plates were coated with rabbit polyclonal antibodies (Boeringer-Mannheim) raised against synthetic β -amyloid (1-40) (Sigma) (100 ng/well) via covalent attachment to epoxy-coated ELISA plates by incubation at 4°C for 16 hours. (Eupergit-C containing epoxy groups, Rohm GmbH, Darmstadt, Germany, using beads or paper as the solid phase Solomon, et al., 1992; Solomon et al., 1993). The residual epoxy groups were blocked by non-fat milk.

The reaction mixtures containing aqueous solution of β -amyloid (100 ng/ml), heparan sulfate (50 mM) and/or chloride metal solutions (10^{-3} M at pH 6.5), was first incubated at 37°C for three hours. The aggregated β -amyloid preparations were removed by centrifugation at 15,000 g for 15 minutes. The residual soluble β -amyloid was incubated for another one hour at 37°C with mAbs AMY 33 and/or 6F3D at equal molar ratio antibody/antigen. In another set of experiments, the mAbs were added to the reaction mixtures before incubation at 37°C and then incubated together for 3 hours at 37°C.

After the incubation period, the immunocomplexed amyloid preparations were added to the ELISA plates, previously coated with polyclonal anti-

amyloid antibodies. The amount of mAb bound will be proportional to the amount of soluble amyloid which remained after exposure to aggregation conditions.

The amount of bound antibody was determined
5 using α -mouse second antibodies labeled with
horseradish peroxidase (HRP). The enzyme activity of
HRP is directly proportional with the amount of
residual amyloid bound to rabbit polyclonal
antibodies. The enzyme activity of HRP was measured
10 using O-phenylenediamine (OPD) as substrate. The
color developed was measured at A_{495} using an ELISA
reader. Data represent the mean of triplicate
determinations. The standard deviation of the intra-
assay and interassays were less than 5% in all cases.

15

Competitive enzyme-linked immunosorbent assay (ELISA)

β -Amyloid peptide (50 ng/well in PBS) was
covalently bound to epoxy-coated ELISA plates
overnight at 4°C. The remaining active groups on the
20 plate were blocked with skim milk.

The reaction mixture containing soluble
 β -amyloid peptide (50 ng in 10 μ l PBS) was first
incubated for three hours at 37°C. Each of the mAbs
was added and allowed to interact with the remaining
25 soluble β -amyloid peptide for one hour at 37°C. The
amount of each antibody was sufficient to bind to all
soluble β -amyloid peptide (50 ng) before first
incubation at 37°C. In parallel assays, the same
amounts of mAbs were added to the β -amyloid peptide
30 solutions before the first incubation at 37°C for
three hours. After the second incubation, the
 β -amyloid preparations centrifuged at 15,000 \times g for
15 minutes and the soluble reaction mixture was
applied onto the ELISA plates previously coated with
35 β -amyloid peptide.

The antibody that does not bind to the insoluble β -peptide removed by centrifugation will bind to the β -amyloid peptide coated on the ELISA plate. The amount of antibody bound to the β -amyloid peptide coated plate will be inversely proportional to the amount of residual soluble β -amyloid peptide. The amount of bound antibody was detected using α -mouse antibodies labeled with horseradish peroxidase (HRP). Degradation of the O-phenylenediamine (OPD) substrate by HRP was monitored at OD₄₉₂ using an ELISA reader. The maximal amount of antibody necessary to bind all the coated β -amyloid peptide in the absence of β -soluble peptide was determined for each mAb and was considered as 100% of mAb bound. The data represent the mean of five replicates.

β -Amyloid Peptide Aggregation and Immunocomplexation

Synthetic β -amyloid peptide 1-40 (A β 1-40) was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Reaction mixture tubes containing 200 μ l of an aqueous solution of β -amyloid peptide (10 mg/ml) pH 6.7 were incubated for three hours at 37°C. Aggregated β -amyloid samples were removed by centrifugation at 15,000 \times g for 15 minutes. Aliquots of residual soluble β -amyloid peptide were then incubated for another 60 minutes with increasing amounts of the appropriate mAbs to produce totally immunocomplexed β -amyloid peptide. In another set of experiments, mAbs at equimolar antibody/antigen concentrations were added to the reaction mixtures before the first incubation period of three hours at 37°C.

Electron Microscopy.

Negatively stained amyloid fibrils were prepared by floating carbon-coated grids on aqueous

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peptide solutions (1-2 mg/ml) and air drying. Fibrils of β -amyloid, either alone or immunocomplexed to mAb AMY-33 (molar ratio 4:1) for three hours at 37°C, were negatively stained with aqueous uranyl acetate (2% wt/vol) and then visualized using a JEOL-1200 Ex electron microscope operated at 80/KV, using a magnification of 25,000.

Fluorimetry.

Fluorimetric analysis of soluble β -amyloid peptide and the immunocomplex with AMY 33 (molar ratio 4:1) stained with Thioflavin T (Sigma Chemical Co., St. Louis, MO., USA) was performed by standard method (Levine, 1993). Fluorescence was measured using a Perkin-Elmer LS-50 fluorimeter at λ_{ex} = 482 nm. The aggregation reaction was followed for seven days at 37°C.

EXAMPLE 1

CPA Model System

Aggregation of heat denatured CPA was followed by determination of the residual enzymic activity of CPA using esterase and peptidase substrates. CPA (1 mg/ml) was incubated at various temperatures for one hour, and residual enzymic activity was determined. The temperature of 50°C was chosen for further study. At this temperature, mAbs studied keep all their immunological activity (unpublished data). Effect of immunocomplexation of CPA with its mAbs was monitored by: (1) Determination of enzymic activity and (2) ELISA measurements as described herein above.

Monoclonal antibodies raised against native antigens proved to be powerful tools in identification and characterization of folding steps by recognition of incompletely folded antigens (Hendrick and Hartl,

1993). The selected antibodies might interact at sites where protein unfolding is initiated, thereby stabilizing the protein and suppressing further aggregation.

- 5 The main difference between mAbs and molecular chaperones is that the latter does not bind to native proteins and is capable of interacting with many different polypeptide chains without exhibiting an apparent sequence preference (Goloubinof et al., 10 1989). Moreover, chaperones suppress aggregation but do not redissolve aggregate proteins already present.

- The aggregation of CPA and loss of its enzymic activity was found to be dependent on the temperature and the time of incubation (Figures 1, 2). 15 Esterase activity seems to be more affected at higher temperature than peptidase activity, indicating that these activities follow different reaction mechanisms (Figure 2). These data are compatible with applicant's previous results (Solomon et al., 1989; 20 Solomon and Balas, 1991), as well as with the findings of Vallee and his collaborators (1969), who postulated that the active site of CPA consists of non-identical but interacting binding sites for peptides and ester substrates. As shown in Figure 2, the immunological 25 recognition of partially heat denatured enzyme is better conserved than its residual enzymic activity.

- The inhibition of CPA aggregation, induced by incubation at 50°C for one hour by its interaction with two mAbs, CP, and CP₁₀, was followed by measuring 30 the peptidase and esterase enzymic activities (Figure 3). The two mAbs, CP₁₀ and CP, were chosen for this study on the basis of previous data regarding their effect on the enzyme behavior (Solomon and Schwartz, 1995; Solomon et al., 1989; Solomon and Balas, 1991). 35 The protection of enzymic activity of heated CPA was dependent on the amount of antibody added to the

- enzyme and a molar ratio of 1:1 antibody/enzyme was sufficient for the maximum protection effect. The peptidase activity of the CPA-CP₁₀ complex was maintained at 90% of its initial activity in the
- 5 presence of mAb CP₁₀. The protective effect of mAbs on CPA activity during heat denaturation was found to be related to the location of the antigenic site of each antibody (Figure 4). Even a great excess of unrelated antibody did not assist in maintaining CPA activity.
- 10 Increase in preservation of enzyme activity can be reached, however, in the presence of a pair of two antibodies. This effect seems to be the result of a "locking" of the conformation caused by simultaneous interaction with two different antibodies at two
- 15 distinct epitopes (Solomon and Balas, 1991).

The amount of aggregated CPA was quantitated by ELISA measurements. Disappearance of CPA, as a result of its aggregation during incubation for one hour at 50°C, was followed by a competitive ELISA

20 assay (Figure 5) and a sandwich assay (Figure 6). The mAb, CP₁₀, maintained 100% of the CPA activity in solution during heating for one hour at 50°C (Figure 6); CP, provided a slight effect on CPA protection at 50°C. Both antibodies prevent the aggregation of CPA,

25 similar to the data shown in Figure 4, recognizing "key positions" on the molecule responsible for heat denaturation and aggregation of CPA.

The biological activity of the enzyme seems to be more sensitive to high temperatures than the

30 insolubilization process. Subtle heat-induced conformational changes occurring in CPA molecules are reflected by change in enzymic activity, even before transition between native-molten globule conformation-aggregated states occurred. These findings are in

35 contradiction to previous suggestions that the

biological function of a protein does not necessarily require fully folded protein (Hattori et al., 1993).

The antigen binding site of mAb CP₁₀ (previously named CP₁₀₀) was identified as one of the immunodominant regions of the enzyme, localized on the surface of the molecule between amino acids 209-218 (Solomon et al. 1989). The localization of the epitope recognized by CP, has not yet been clarified, but it does not interfere with the mAb CP₁₀ during simultaneous binding to CPA molecule, as suggested by additivity measurements (Solomon and Balas, 1991).

Similar effects in suppression of antigen aggregation were obtained after immunocomplexation of horseradish peroxidase.

The data available in the literature of antibody preparation suggests that for practically all antigens it should be possible to prepare monoclonal antibodies and by means of the present invention it will be possible to select those monoclonal antibodies which bind with high affinity without affecting the target molecule activity. Moreover, mAbs like the majority of immunoglobulins, are robust molecules and survive in a variety of environments, including high temperatures, low pH, denaturing agents. Formation of such immunocomplexes should provide a general and convenient method for suppression of aggregation and stabilization of their antigen without affecting the biological properties of the given antigen as shown also in the following Examples.

EXAMPLE 2

Amyloid System

This example investigates the immunocomplexation effect on the *in vitro* aggregation of β -amyloid. Aggregation of β -amyloid was found to be dependent on the pH, peptide concentration,

temperature and time of incubation (Burdick et al., 1992). In applicant's experiments, the aggregation of β -amyloid was performed by incubation of an aqueous solution of β A4 (10 mg/ml) for three hours at 37°C.

- 5 The β -amyloid aggregation was followed by ELISA measurements using two different commercially available monoclonal antibodies raised against β -amyloid: α -human β -amyloid 6F/3D obtained from Accurate Chemical and Scientific Corp, Westbury, N.J.
- 10 USA, and mAb AMY 33 (Stern et al., 1990), purchased from Zymed, San Francisco, CA, USA, raised against peptides 8-17 and 1-28, respectively, of the β -amyloid.

- The addition of the antibodies was made
- 15 before or after exposure of synthetic β -amyloid to the aggregation process (Figs. 7A, B). The aggregation of the β -amyloid was performed in the presence of heparan sulfate and/or metal ions, such as Zn^{2+} and Al^{3+} . The antibody AMY-33, which recognizes an epitope spanning
- 20 the amino acids 1-28 of the β -amyloid sequence, inhibits the β -amyloid aggregation occurring in the presence or absence of heparan sulfate (Figure 7A). Any significant effect on metal-induced amyloid
- 25 aggregation was observed under the same experimental conditions. The mAb 6F/3D, recognizing an epitope located in amino acids 8-17 of β -amyloid, interferes with Zn^{2+} -induced aggregation, showing a partial solubilization effect on already aggregated β -amyloid, but has no effect on other aggregating agents (Figure
- 30 7B).

- Metals, such as Zn^{2+} and Al^{3+} , have been proposed as risk factors for Alzheimer's disease development (Mantyh et al., 1993; Frederickson, 1989; McLachlan et al., 1991). The aggregation of β A4
- 35 induced by aluminum is distinguishable from that induced by Zn in terms of role, extent, pH and

temperature dependence (Mantyh et al. 1993). Although the precise site of interaction of metal ions and β A4 is not clarified, several residues in β A4 are candidates for metal binding. The β A4 histidine residues (His₁₃-His₁₄) may be implicated in fibril formation and it is conceivable that at least His₁₄ remains available for intermolecular electrostatic interactions between anti-parallel chains (Talfous et al., 1994). The site defined by Val₁₂-His₁₃-His₁₄-Glu₁₅-Lys₁₆-Leu₁₇ has been identified as a sequence containing a heparan sulfate binding domain (Fraser et al., 1992) and His₁₃ and Lys₁₆ are supposed to provide the cationic binding sites being exposed on the same face of the peptide β sheet (Talaious et al. 1994).

Binding of mAb AMY-33 to β A4 prevents self-aggregation of the β -amyloid. This antibody prevents intramolecular aggregation occurring in the presence of heparan sulfate, which is supposed to affect only the aggregation of preexisting amyloid fibers (Fraser et al., 1992). Inhibition of β -amyloid aggregation in the presence of mAb 6F/3D was partially effective only in the presence of Zn²⁺.

EXAMPLE 3

Amyloid Electron Microscopy and Fluorimetric Studies

Electron microscopy of negatively stained β -amyloid and its immunocomplex with mAb AMY-33 revealed that even at a low peptide/antibody ratio, fibrillar β -amyloid was converted to an amorphous state. Thioflavin T, a suitable probe for detecting the fibrillar aggregation of β -amyloid peptide, confirmed the electron microscopy results. Dilution of the peptide directly from water into dye-containing buffer had no effect on dye fluorescence. In the presence of aggregated A β 4 (1-40), however, a change occurred in the excitation spectrum of Thioflavin T,

manifested as a new peak at 450 nm that was not seen with the free dye (Figs 8A, B.) Aggregated β -amyloid peptide enhances the fluorescence emission of Thioflavin T at 482 nm as a function of

- 5 incubation-time. Adding mAb AMY-33 (4:1) to the A β 1-40 solution before exposure to 37°C prevented the increase in fluorescence at 482 nm, whereas the addition of unrelated antibody did not interfere with the fluorescence peaks.

10

EXAMPLE 4

Comparative Studies of Monoclonal Antibodies

- Monoclonal antibodies were added to the reaction mixture before or after incubation of
- 15 synthetic β -amyloid peptide at 37°C for three hours. The results shown in Figure 9 indicate that mAbs 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β -amyloid peptide, inhibited the formation of β -amyloid by 90% when
- 20 compared with aggregation occurring in the absence of the respective antibodies. The mAb AMY-33, which recognizes another epitope located within residues 1-28, affected the self-aggregation of β -peptide to a lower extent of approximately 40%. The antibodies,
- 25 2H3 and 1C2, directed to the regions comprising peptides 1-12 and 13-28, respectively, had a considerably lower effect on *in vitro* amyloid formation.

- On the basis of applicants findings
- 30 regarding these Examples and other antigen-antibody systems studies (Solomon et al., 1989; Solomon and Balas, 1991), the formation of the immunocomplexes with selected, highly specific monoclonal antibodies, should provide a general and convenient method to
- 35 prevent aggregation of the proteins without affecting their biological properties.

At least 15 different polypeptides are known to be capable of causing *in vivo* different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils.

5 Recent advances in antibody engineering technology, as well as in the development of suitable delivery systems (Haber, 1992; Pluckthun, 1992; Travis, 1993; Marasco et al., 1993) make it possible to develop functional small antibody fragments to
10 serve as therapeutic chaperones for the treatment of Alzheimer's disease as well as other human amyloidosis diseases by gene based therapies.

 Application of the above findings for *in vivo* aggregation, can confer to single chain
15 antibodies (Pluckthun, 1992) or other engineered antibody fragments, a protective role in the renaturation of recombinant proteins.

 Throughout this application various publications are referenced by citation or number.
20 Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to
25 which this invention pertains.

 The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of
30 limitation.

 Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the
35 invention may be practiced otherwise than as specifically described.

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CLAIMS

What is claimed is:

- 5 1. A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a native target molecule epitope with a high binding constant and are non-inhibitory to the biological activity of the target molecule by
- 10 denaturing a target molecule which aggregates,
- mixing the target molecule with a presumptive anti-aggregation molecule,
- 15 incubating the mixture under conditions allowing for aggregation,
- selecting non-aggregated mixtures, and testing the nonaggregated target molecule coupled to the anti-aggregation molecule for
- 20 bioactivity thereby selecting an anti-aggregation molecule with chaperone-like activity.
2. The method of claim 1 wherein the anti-aggregation molecule is a monoclonal antibody, a
- 25 genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen on the antibody.
3. The method of claim 1 wherein the anti-
- 30 aggregation molecule is a single chain monoclonal antibody.
4. The method of claim 1 wherein the target molecule is β -amyloid.

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5. The method of claim 1 wherein the target molecule is a prion protein.

6. A method of selecting anti-aggregation
5 molecules with chaperone-like activity that have characteristics including binding to a target molecule epitope with a high binding constant, reversing aggregation effects and are non-inhibitory to the biological activity of the target molecule by
10 preparing an aggregated target molecule, mixing the target molecule with a presumptive anti-aggregation molecule, selecting mixtures with non-aggregated target molecules, and
15 testing the target molecule coupled to the anti-aggregation molecule for bioactivity thereby identifying an anti-aggregation molecule with chaperone-like activity.

20 7. The method of claim 6 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen on the antibody.

25 8. The method of claim 6 wherein the anti-aggregation molecule is a single chain monoclonal antibody.

30 9. The method of claim 6 wherein the target molecule is β -amyloid.

10. The method of claim 6 wherein the target molecule is a prion protein.

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11. The method of treating a protein aggregation disease including the steps of preparing at least one anti-aggregation molecule that binds to an aggregating protein which is the cause of a disease and which prevents aggregation while allowing bioactivity;

creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the anti-aggregation molecule that binds to an aggregating protein and which prevents aggregation while allowing bioactivity; and administering the expression vector.

12. The method of claim 11 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen.

13. The method of claim 12 wherein the monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is a single chain monoclonal antibody.

14. The method of claim 12 wherein the monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is a human anti- β -amyloid monoclonal antibody.

15. The method of claim 12 wherein the monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is an anti-prion protein monoclonal antibody.

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16. The method of claim 11 wherein at least two anti-aggregation molecules are used.

17. The method of claim 11 wherein the
5 expression vector is a virus.

18. The method of claim 17 wherein the expression vector is a disable retro virus.

19. The method of claim 17 wherein the
10 expression vector is a retroviral shuttle vector.

20. The method of claim 17 wherein the expression vector is vaccinia virus.
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21. The method of claim 17 wherein the expression vector is an adenovirus.

22. The method of claim 17 wherein the
20 expression vector is a plasmid.

23. A pharmaceutical composition comprising an expression vector comprising nucleic acid including a sequence which encodes in expressible form an anti-
25 aggregation molecule that binds to an aggregating protein while allowing bioactivity and a pharmaceutically acceptable carrier.

24. The expression vector as set forth in
30 claim 23 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen.

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25. The expression vector as set forth in claim 23 wherein the anti-aggregation molecule is a single chain monoclonal antibody.

5 26. The expression vector as set forth in claim 24 wherein the monoclonal antibody is a human anti- β -amyloid monoclonal antibody.

10 27. An expression vector for use as a therapeutic agent which comprises nucleic acid including at least one sequence which encodes in expressible form an anti-aggregation molecule that binds to an aggregating protein which is associated with a disease.

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28. The method of treating a protein aggregation disease including the steps of preparing at least one human monoclonal antibody that binds to an aggregating protein which is the cause of a disease and allows bioactivity when bound, and administering the monoclonal antibody.

25 29. The method of claim 28 wherein the aggregating protein is β -amyloid.

30 30. The method of claim 29 wherein the monoclonal antibody is an anti- β -amyloid and is selected from the group consisting of AMY-33 which recognizes an epitope spanning amino acids 1-28 of β -amyloid and monoclonal antibodies 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β -amyloid peptide.

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SUBSTITUTE SHEET (RULE 26)

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31. A method of improving solubility and yields in production of recombinant proteins by preparing an expression vector containing a genetic sequence for an anti-aggregation molecule that
5 suppresses the kinetics of aggregate formation while still encouraging formation of native protein structure, and favors the desired folding reaction of a recombinant protein,

co-transforming a host cell with the
10 expression vector for an anti-aggregation molecule and an expression vector for a recombinant protein thereby increasing intracellular production of the fusion proteins and preventing formation of inclusion bodies and facilitating extracellular protein secretion.

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SUBSTITUTE SHEET (RULE 26)

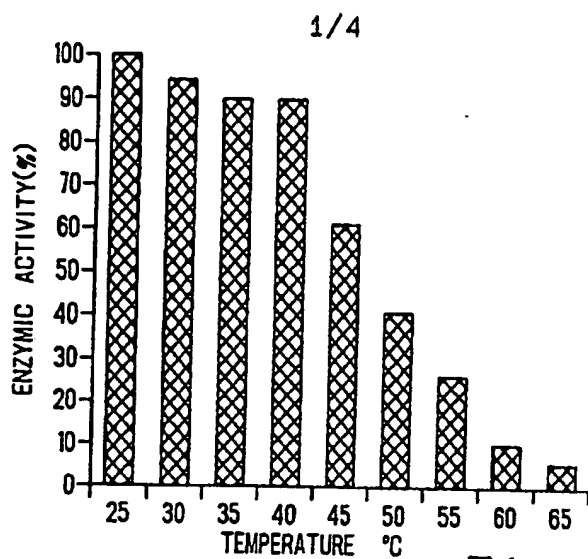


Fig-1

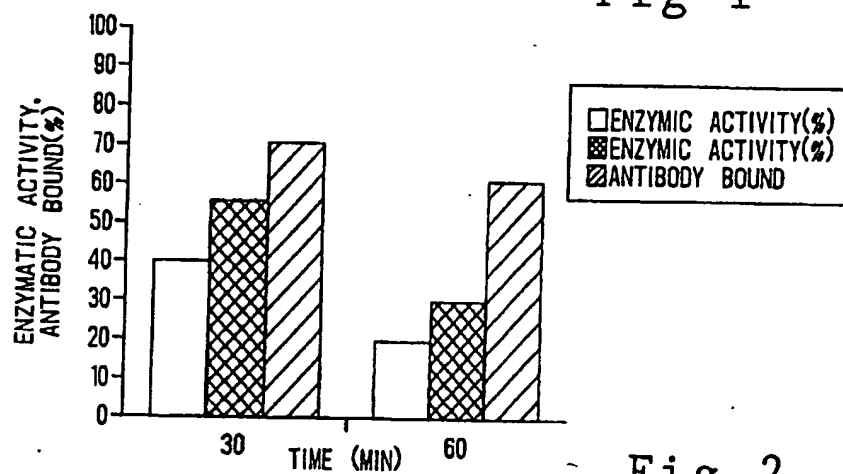


Fig-2

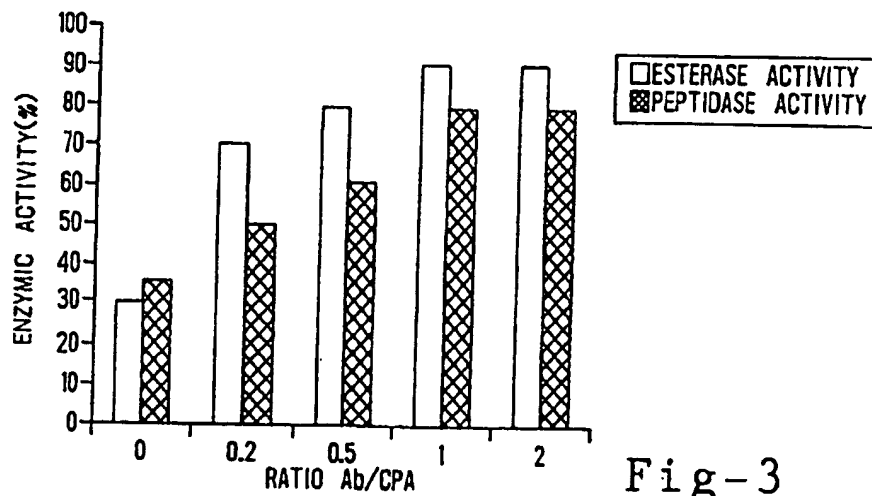


Fig-3

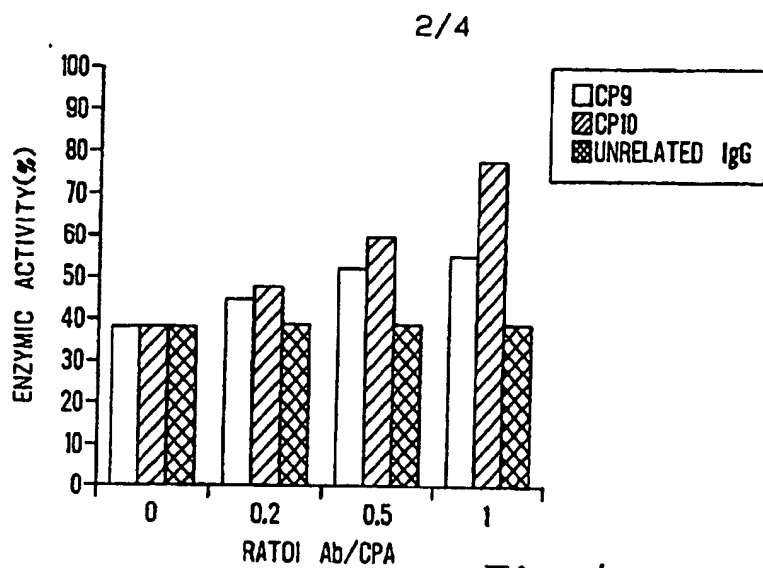


Fig-4

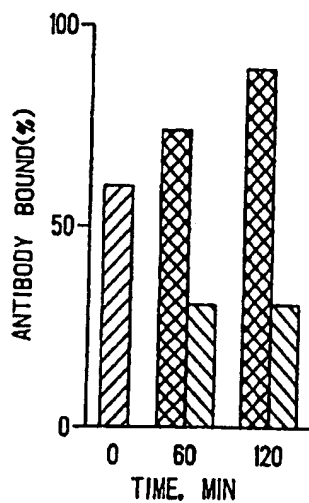


Fig-5

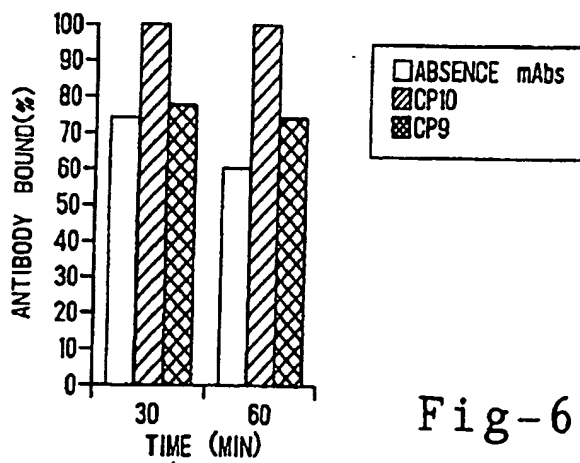
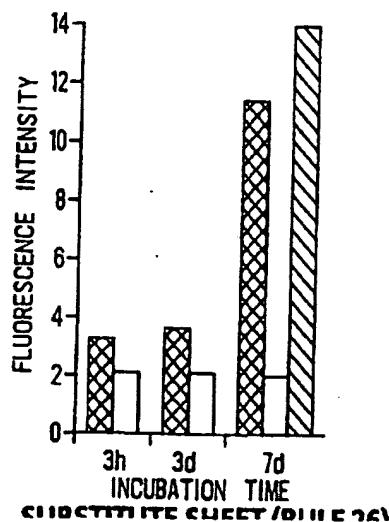
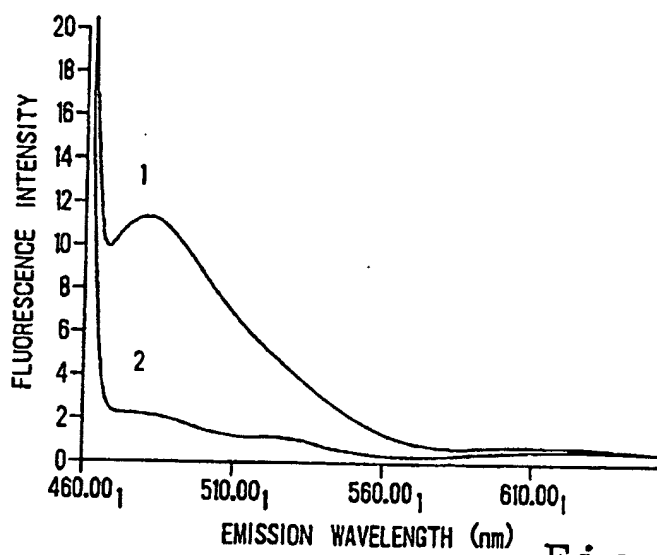
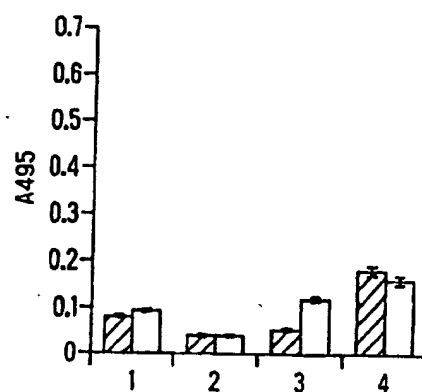
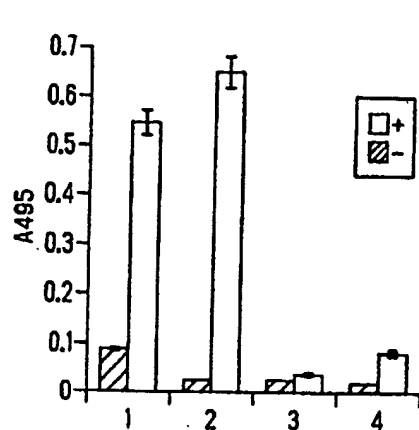


Fig-6



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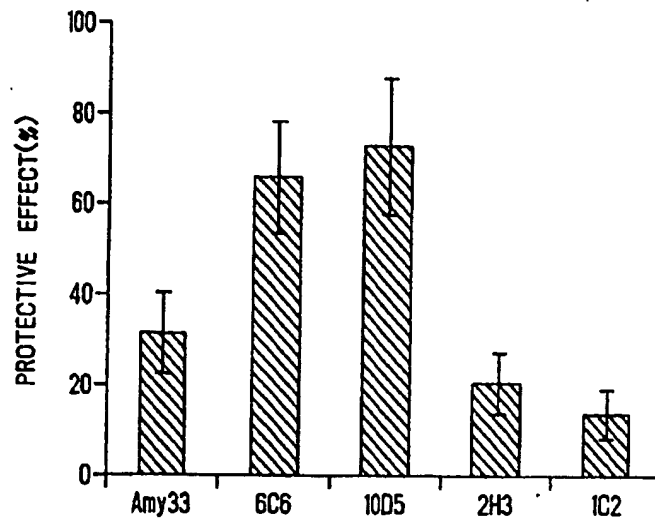


Fig-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16092

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53; A61K 38/00, 31/66; C12N 15/00; C12P 21/06

US CL : 435/7.1, 69.1, 320.1; 514/12, 130

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 320.1; 514/12, 130

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| X | Biochemical and Biophysical Research Communications, Volume 192, No. 2, issued 30 April 1993, Wisniewski et al, "Apolipoprotein E: Binding to Soluble Alzheimer's β -Amyloid", pages 359-365, especially pages 362-363. | 1-30 |



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" documents referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A"

document member of the same patent family

Date of the actual completion of the international search

03 APRIL 1996

Date of mailing of the international search report

23 APR 1996

Name and mailing address of the ISA/US
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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16092

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/16092

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-10, drawn to a method of selecting anti-aggregation molecules.

Group II, claim(s) 11-21 and 27-29, drawn to a method of treating a protein aggregation disease.

Group III, claim(s) 22-26, drawn to a pharmaceutical composition and an expression vector.

Group IV, claim(s) 30, a method to improve solubility and yields in production of recombinant proteins.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: A special technical feature does not link the claims as the prior art reveals compounds which prevent protein aggregation (Wimniewski et al (1993) Biochem. Biophys. Res. Comm., 192, 359-365. There is no unity of invention absent a special technical feature which links the claims as provided in PCT Rule 13.2.

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